VARIATIONS IN THE NUMBER AND SIZE OF NUCLEI IN THE CELLS OF THE KIDNEY TUBULES OF AN AUSTRALIAN DESERT FROG, CYCLORANA (CHIROLEPTES) ALBOGUTTATUS (GÜNTHER)

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FIVE FIGURES

These observations on variations in the number and size of the nuclei in the cells of kidney tubules were made incidentally to a histological examination of the glomerular condition in certain species of Australian desert frogs. Previous reports (Sweet, '07; Baldwin, '37) had indicated that the glomeruli, in some forms, were either greatly reduced in number and size or were entirely absent. The glomerular situation will be the subject of a separate report. The renal nuclear phenomena encountered were quite unexpected and the picture presented is equally as complicated as that found in the mammalian liver (Wilson and Leduc, '48).

MATERIALS

Two species of the Australian desert frog were made available through the courtesy of Mr. Arthur Loveridge, Curator of Reptiles and Amphibians, Museum of Comparative Zoölogy, who permitted the author to dissect out the kidneys. One specimen of Cyclorana (Chiroleptes) platycephalus (male, anterior nares to anus, 62 mm) and two specimens of Cyclorana (Chiroleptes) alboguttatus (females, anterior nares to anus, 56 and 62 mm, respectively) were studied.

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Of these only one specimen of Cyclorana alboguttatus showed extensive departures from the usual nuclear pattern and most of the descriptions and all of the illustrations are based on this frog. The state of histological preservation of the kidneys was remarkably good.

Nothing is known about the fluid originally used to preserve these animals. They were stored in 70% alcohol at the time they were secured for dissection. The specimen of Cyclorana platycephalus was collected 50 miles northeast of Yalgoo, Western Australia and was obtained by the Museum of Comparative Zoölogy by exchange in 1931. The animal was intact. Specimen A of Cyclorana alboguttatus, on which this paper is primarily based, was collected in the Cox River Valley, New South Wales in 1930 and was originally deposited in the Queensland Museum. The abdominal cavity had not been opened. Specimen B also came from Queensland Museum through exchange in 1915. The locality from which it was collected was not recorded. The abdominal cavity had been opened at some unknown time. Transverse, serial sections, 6 μ thick, were made of all three kidneys. They were stained routinely in hematoxylin and eosin, following treatment from 24 to 48 hours with a 3% solution of potassium bichromate. In Cyclorana platycephalus the nuclear stain was rather diffuse but the regional differentiations of the tubules were readily recognized. In both specimens of alboguttatus the nuclear stain was comparable to that of recently fixed tissue. In specimen A the cytoplasm of the tubules appeared relatively homogeneous but the brush border on the cells of the proximal convoluted segments could be recognized. The erythrocytes were hemolyzed but the cilia on the peritoneal funnels were distinctly shown. In specimen B there was evidence of considerable shrinkage but the general pattern of fixation seemed about as good as that obtained with Helly's fluid. The erythrocytes were not hemolyzed, the distal convoluted segments appeared rodded, and in the proximal convoluted tubules both brush border and cyto-

plasmic granules were well preserved. The chromaffin cells of the adrenal bodies were selectively stained.

Unfortunately, there were no records to indicate the time of year or the conditions under which the animals had been collected. Both species (Sweet, '07; Buxton, '23) make permanent burrows, store up water, and aestivate during periods of drought. Whether or not exposure to periodic desiccation is in any way related to the nuclear phenomena in the kidney cannot be determined. Specimen A of Cyclorana alboguttatus may be an exceptional animal, although the kidneys of the other two animals contained many binucleate cells. The paper of Sweet ('07) who made a rather detailed study of the alboguttatus kidney contains no reference to the nuclei of the tubules. The conclusion must be that the nuclear size and number were normal in the specimens she studied since the extreme nuclear picture seen in specimen A is too striking to be overlooked, or if observed, not to be mentioned.

DESCRIPTION

In the topographical relationship of the uriniferous tubules the kidneys of these Australian species conformed rather closely to the general anuran plan (Stewart, '27), with the several segments of the tubular units segregated into three rather definite and approximately crescentic zones. A ventral zone consisted almost entirely of closely coiled, distal convoluted segments, and ciliated peritoneal funnels which opened into the renal veins. Above in an intermediate and less definite zone the glomeruli and the connecting portions of the tubules (necks to initially straight, proximal convoluted tubules; distal convoluted segments to initial collecting tubules) were found. The definitive proximal convoluted segments were uniformly restricted to a relatively wide, dorsal zone, in which were also found short vertical ducts which joined the longer transverse collecting tubules leading laterally into the Wolffian duct.

Cyclorana alboguttatus, specimen A

Binucleate and quadrinucleate cells. Binucleate and quadrinucleate cells were found in greatest numbers in the portions of proximal convoluted tubules that were situated most dorsally, i.e., close beneath the kidney capsule. They occurred much less frequently toward the junction of the proximal convoluted with the distal convoluted segments. Intermediate segments were not recognized. Quadrinucleate cells were entirely restricted to the proximal convoluted tubules but binucleate cells were occasionally also found in distal convoluted and even in collecting tubules. The majority of the nuclei tended to be of uniform size and shape, quite comparable to those in ordinary uninucleate cells, presumably normal diploid units (fig. 1). The predominant form was spherical. The chromatin was relatively dispersed and a conspicuous nucleolus was seen in almost all cells. However, ellipsoidal nuclei were not uncommon (fig. 2). In a limited number of binucleate cells the nuclei were relatively large, at least double the usual volume (figs. 3 and 5). Quadrinucleate cells with such large-sized nuclei were not observed. The size of the cells appeared to be directly related to the amount of nuclear material present but the walls of the tubules were not appreciably thickened except in regions where quadrinucleate cells were locally grouped (fig. 2).

In binucleate cells the nuclei were usually closely apposed to one another and the pairs commonly lay parallel to the free surface of the cell; occasionally they were oriented in a perpendicular manner. In quadrinucleate cells the nuclei were variously arranged. They were sometimes closely spaced in a straight line. In other cases they overlapped each other slightly, giving a staggered effect (fig. 2). More commonly, they were symmetrically placed to form a square or rectangle (fig. 2) but they were never found scattered irregularly throughout the cytoplasm. The groups of 4 nuclei usually lay in a plane parallel with the free surface of the cell.

Trinucleate cells. The normal expectancy with regard to nuclear number, especially if the nuclei are of equal size is that they would occur in groups of 2, 4, 8, etc. In the kidney three nuclei of equal size within a single cell were frequently seen when the plane of the section was parallel with or at right angles to the long axis of the tubule. However, in such cases there is always the possibility that a 4th nucleus might be present in an adajcent section. This is difficult to verify since the plane of the section might pass through the membrane of the adjoining cell and the additional cell go unrecognized. More reliable observations can be made on tangential sections where the clustered nuclei tend to lie in a plane parallel to the free surface of the tubule, and the cell boundaries other than those of the free and attached surfaces can be readily recognized. In such sections the appearance of three nuclei of equal size in a single cell was rare. All such trinucleate pictures probably represent incomplete sections of quadrinucleate cells.

However, other trinucleate cells, apparently variants of the quadrinucleate condition, were quite common. In these cells, there was one larger nucleus accompanied by a pair of normal size (figs. 3 and 5). The three nuclei may be either arranged in a row or the pair closely applied to the larger member of the group. In one instance the larger nucleus was constricted as if in the process of amitotic division, but it could equally as well be interpreted as a product of incomplete fusion of two nuclei of normal size. The constricted nucleus contained two nucleoli, one in each lobe, and was oriented with its long axis perpendicular to the normal-sized pair.

Other multinucleate cells. An extensive search was made for cells containing more than 4 nuclei but only two were found. In one instance, in a medial longitudinal section of a tubule, an elongated cell was found in which 6 nuclei of basal size were in evidence. These were arranged in an irregular row parallel with the tubular lumen and overlapped sufficiently to give a slightly staggered appearance to the chain of nuclei.

Their compact arrangement indicated that they were contained in a single cytoplasmic mass, but it was not possible to identify with certainty any portion of this multinucleated unit in adjoining sections. Accordingly, it is not possible to state that this cell contained only 6 nuclei. In another cell there were 5 nuclei of basal size grouped in a circle. This was in an oblique, tangential section through a convolution and the cell was obviously incomplete, but it was not possible to identify the missing portions in adjoining sections.

Cells with single, very large nuclei. Large cells with single large nuclei were rarely encountered, usually not more frequently than one in an examination of 5 complete cross sections of a kidney (fig. 4). The volume of such nuclei was many times that of nuclei of basal size and they probably represent at least an octoploid condition. The nucleolar patterns were not sufficiently clear in any of these nuclei to permit a determination of either their number or relative sizes.

Accessory chromatic vesicles. Another feature of interest, besides the variations in size and number of well-constituted nuclei, is the occasional occurrence of varying numbers of accessory chromatic vescicles; these vary in number from one to at least 4, and were found in close association with nuclear groups and in cells with single large nuclei. They possessed a delicate chromatic network and a distinct membrane. These accessory bodies are suggestive of a failure of some chromosomes or parts of chromosomes to be incorporated into major nuclear units. An alternative explanation would be that they are results of nuclear degeneration and fragmentation, but the large units appear normal and there is little evidence of nuclear regression in the kidney tubules. They were not observed in normal uninucleate cells. Some of the variations in size of apparently homologous nuclear units might be attributed to incomplete incorporation of chromosomes into telophase nuclei.

Evidence of mitotic activity. No advanced stages of mitosis were observed, but a small number of nuclei in progressive

stages of prophase were present in uninucleate cells of the proximal convoluted tubules. These nuclei were only slightly larger than those of ordinary resting uninucleate cells, and were characterized by the disappearance of the nucleolus and the loss of the dispersed appearance of the chromatin. In all, the chromatin was increased in amount and was more densely stained. Quite frequently it was gathered into coarse, granules uniformly spaced throughout the nucleus. In other cases the chromatin granules were arranged into convoluted threads of varying thickness. The thicker threads tended to be concentrated at the nuclear surface but the nuclear membrane was always intact. In isolated cases some crenation was observed but this may have been an artefact of fixation. No free chromosomes were ever seen. These prophase changes were not apparent in any nuclei other than those of normal-sized, uninucleate cells. In rare instances two small, densely-stained nuclei were observed in a single cell. The chromatin was compactly arranged, and no nucleoli could be distinguished. Their appearance suggested that they were daughter nuclei in very late telophase, but no intermediate stages between this condition and prophase were found.

This kidney of specimen A of Cyclorana alboguttatus seems exceptional in possessing so many binucleate, trinucleate and quadrinucleate cells. Binucleate cells were relatively common in specimen B of this species, but no trinucleate cells and only two examples of quadrinucleate cells were found. In the single specimen of Cyclorana platycephalus the only variation in nuclear number was the binucleate condition and these were relatively few even when compared with specimen B of alboguttatus.

DISCUSSION

The nuclei of the cells of the proximal convoluted tubules of Cyclorana alboguttatus may be grouped, on the basis of size, into at least three categories, representing those of minimum volume, presumably diploid, and two other progressively larger classes. Since there is considerable varia-

tion in the size of the individual nuclei within these apparent size-categories it is not possible, in the absence of any typical mitotic figures, to relate directly the differences in size of these larger forms to definite degrees of polyploidy. The normal expectancy would be to find a progressive series of tetraploid, octoploid, (etc.) nuclei whose volumes would vary according to a similar geometric series.

However, variations in nuclear volumes may result from other factors than degrees of suppression, or failure, of normal mitotic activity. The degree of interphasic growth, the rapidity of successive, successful mitoses and specific functional activity of the cell may directly influence nuclear size as shown by Schreiber and Angeletti ('40) in the liver of the growing carp. Here both volume decrease and increase succeed each other in a discontinuous manner. Comparable changes in nuclear volume in the endometrium, and myometrium of the rat have been reported by Salvatore and Schreiber ('47). Striking differences in nuclear volume, related primarily to specific differentiation and functional activity of 36 different cell types in human tissues have also been described by Jacobj ('35). This is also illustrated in the frog kidney where the nuclei of the cells of the neck are small and pyknotic and bear little resemblance in either shape, size or chromatic pattern to those of the convoluted tubules.

Nevertheless in such tissues as the mammalian liver, and in this specific instance the frog kidney, the general pattern of variation in both nuclear size and number favors the interpretation that these conditions stem primarily from varying degrees of failure of the mitotic process (Dawson, '40), possibly complicated by the occurrence of amitosis, nuclear fusion, or even cell fusion. It would seem unprofitable to discuss this problem further since no mitotic figures were available to permit an analysis of either chromosomal numbers or the history of spindle formation. The general problem of the occurrence and formation of binucleate and multinucleate cells and polyploid nuclei in the mammalian liver has been recently critically reviewed by Wilson and

Leduc ('48) and there is little that the author can add to their discussion.

Wide variations in the size and number of nuclei or even the presence of binucleate cells in kidney cells of the Amphibia apparently have not previously received much attention. In fact no references to such conditions have been found in the literature, even in publications in which the amphibian kidney has been discussed at some length (von Möllendorff, '30, Noble, '31). However, even a casual study of slides supplied by the General Biological Supply House, Inc. indicates that a limited number of binucleate cells are also present in the proximal convoluted tubules of the lamprey, shark and frog (species unidentified).

SUMMARY

Variations in the number and size of nuclei in the cells of the tubules of the kidneys of two species of Australian desert frog (Cyclorana alboguttatus [two specimens] and platycephalus (one specimen) are described. In addition to large cells with very large single nuclei an exceptionally large number of binucleate, trinucleate and quadrinuceate cells were found in the proximal convoluted segments of one specimen of alboguttatus. In the other two kidneys only binucleate cells occurred in any considerable numbers. The nuclei, on the basis of size, may be grouped into at least three categories representing those of normal size, presumably diploid, and two other progressively larger classes. In the absence of mitotic figures the degree of polyploidy in such large nuclei could not be determined.

It is suggested that these variations in nuclear number and size, in kidney cells, as in mammalian liver cells, are primarily the result of varying degrees of failure of the mitotic process, possibly complicated by the occurrence of amitosis, nuclear fusion or even cell fusion; the three latter phenomena being regarded as secondary or regulatory in nature.

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PLATES

DESCRIPTION OF PLATES

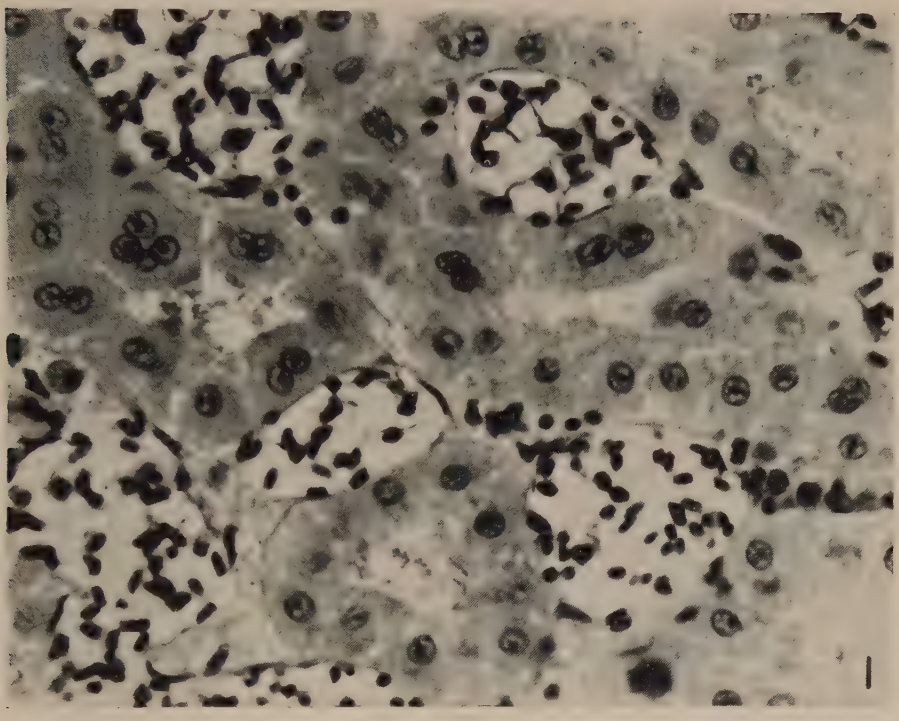
All figures are photomicrographs of segments of proximal convoluted tubules taken at a uniform magnification (× 600). Sufficient depth of focus to include nuclei lying at slightly different levels in the cells was obtained by using a Leitz achromatic, 8 mm oil-immersion objective (× 22) with an aplanatic ocular (× 20). All material was sectioned at 6 μ and stained with hematoxylin and eosin. The intensity of the staining with eosin varied locally and this is reflected in the uneveness in density of the photographs. There is little connective tissue about the tubules and the venous sinusoids are crowded with hemolyzed erythrocytes. All illustrations are from specimen A, Cyclorana alboguttatus.

PLATE 1

EXPLANATION OF FIGURES

1 Cross and partially tangential sections of convoluted tubules showing many ordinary uninucleate cells, several binucleate cells and one quadrinucleate cell. The majority of the nuclei are of uniform size and spherical in form. Larger, ellipsoidal nuclei are seen in one binucleate cell (slightly above and to the right of center of the figure).

2 Tangential section through the wall of a convoluted tubule showing two quadrinucleate and several binucleate cells. In one quadrinucleate cell all 4 nuclei are ellipsoidal, in the other they are spherical. A portion of a distal convoluted tubule is shown in the lower right corner of this figure.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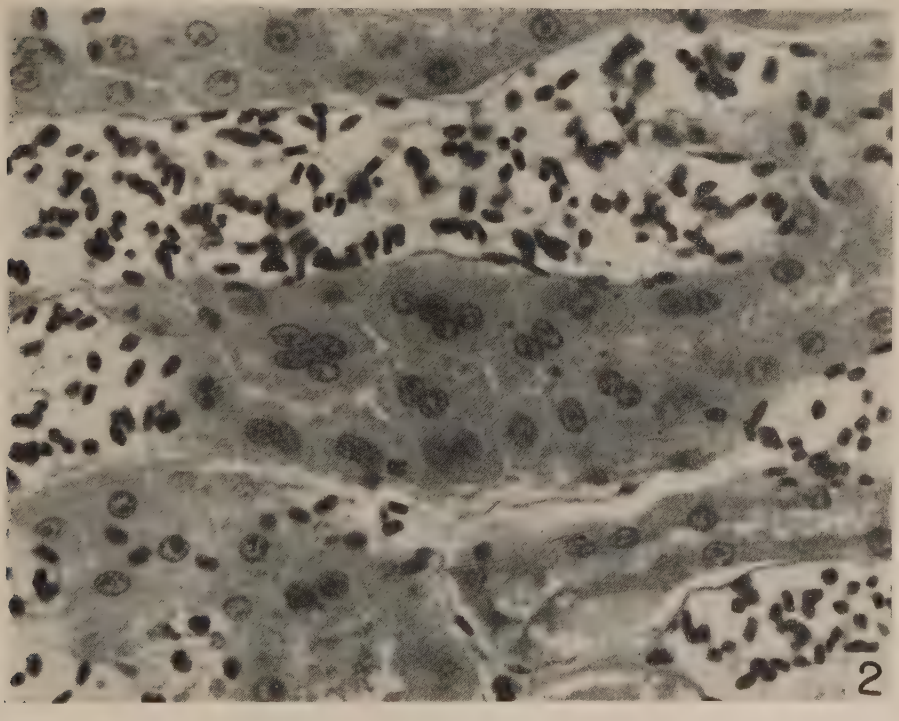
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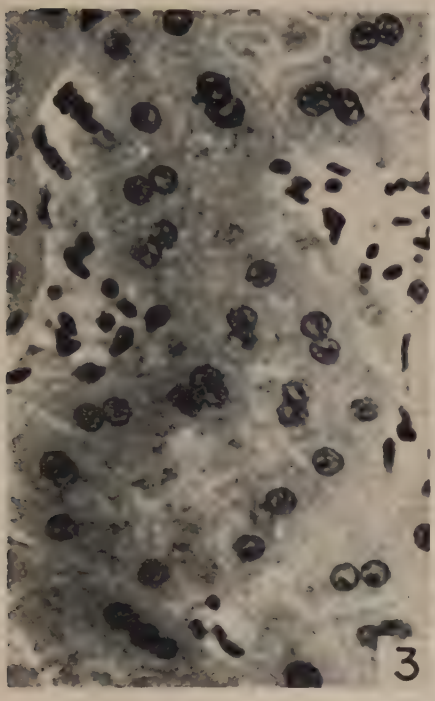
PLATE 2

EXPLANATION OF FIGURES

3 Longitudinal and tangential section of a convoluted tubule. Near the top of the figure there are two binucleate cells with relatively large nuclei. At the bottom a trinucleate cell, with one large and two smaller nuclei, is shown.

4 A section of a convoluted tubule showing a cell with a single, very large nucleus.

5 Across the top of this figure passing from left to right three different nuclear conditions are shown in a convoluted tubule: (1) a trinucleate cell with one larger and two smaller nuclei (2) a binucleate cell with two relatively large nuclei and (3) a cell with a single bilobed nucleus, suggesting a fusion along their major axes of two ellipsoidal nuclei.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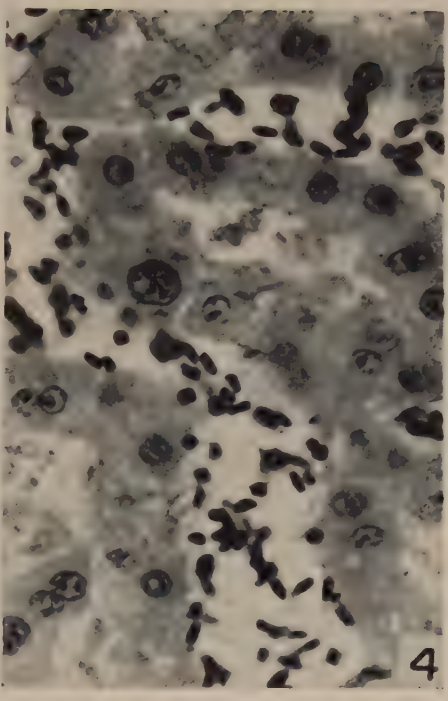
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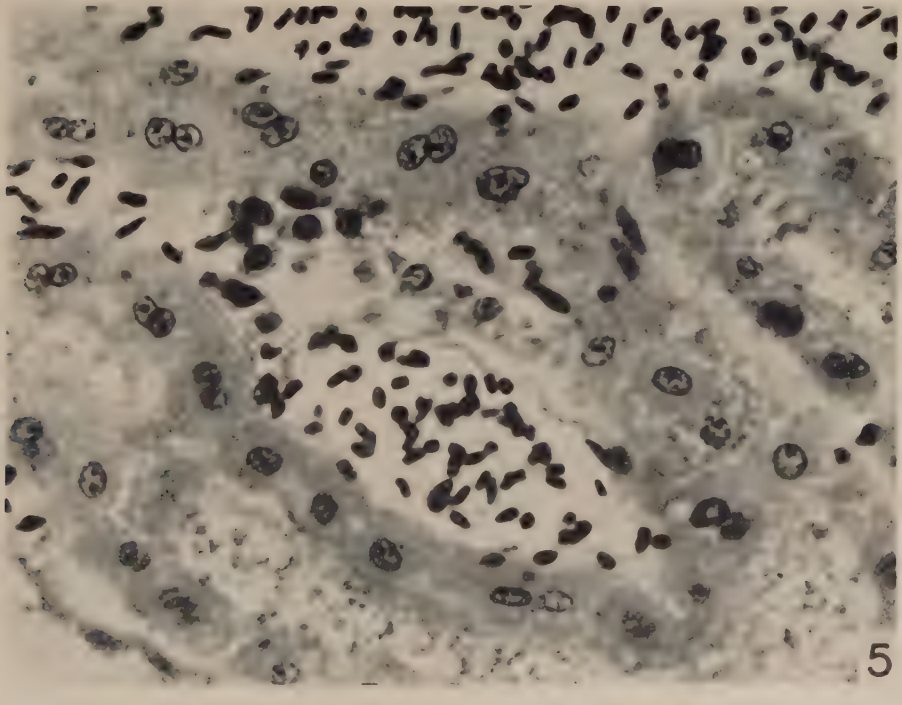
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THE SEGMENTAL DISTRIBUTION OF THE CUTANEOUS NERVES IN THE LIMBS OF MAN

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TWENTY-SEVEN FIGURES

INTRODUCTION

The segmental distribution of the cutaneous nerves in the limbs of man has been the subject of considerable confusion and uncertainty, due to limitations of technique and lack of well controlled human cases for study. A new approach to this problem has been furnished by the many clinical cases in which herniation of the nucleus pulposus of an intervertebral disc compresses a single nerve root intraspinally and is operated for relief (fig, 1), This common cause of nerve root pain radiating down a limb, previously called sciatica, was first clearly identified by Mixter and Barr ('34) and has since become generally recognized by report of many operated cases. Surgical demonstration that this discrete nucleus pulposus herniation commonly compresses only one nerve root, and the finding of an area of diminished cutaneous sensitivity for this functional loss (Keegan, '43, '44, '47) has permitted the drawing of new dermatome charts of the limbs of man and these have been combined with standard dermatomes of the trunk to form a new dermatome chart of the human body (fig. 2). This chart differs significantly from those of previous investigators in that it presents a regular pattern of serial dermatomes in the limbs extending as con-

tinuous bands from the dorsal midline of the body down the arm and the leg. This finding and report is based on 165 cases of dermatome hypalgesia of the upper limb, 47 of which have been verified by surgery, and 1264 cases of dermatome hypalgesia of the lower limb, 707 of which have been verified

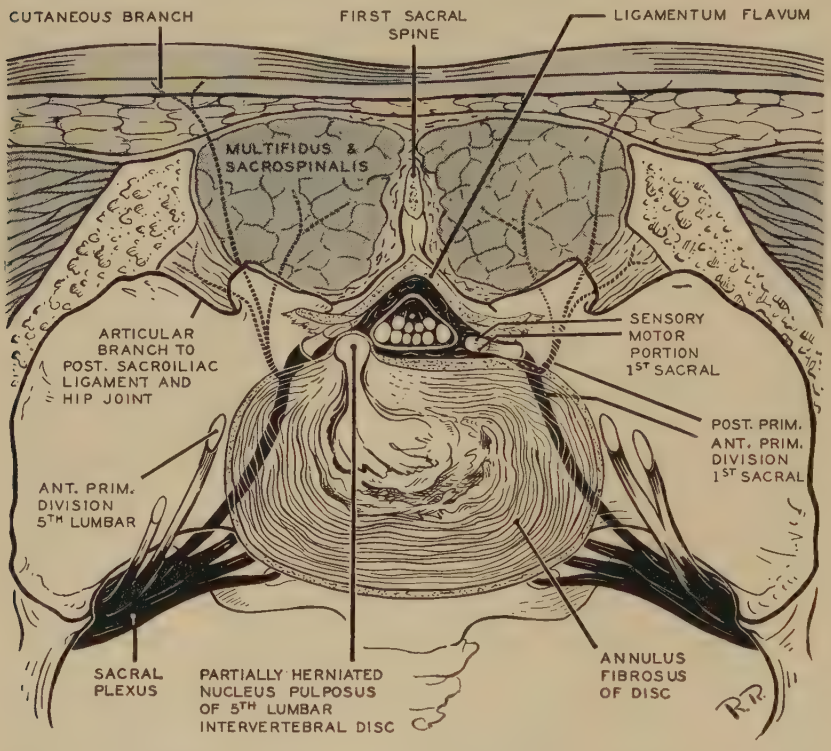
CUTANEOUS BRANCH
FIRST SACRAL SPINE
LIGAMENTUM FLAVUM
MULTIFIDUS & SACROSPINALIS
ARTICULAR BRANCH TO POST. SACROILIAC LIGAMENT AND HIP JOINT
SENSORY
MOTOR PORTION 1ST SACRAL
POST. PRIM. ANT. PRIM. DIVISION 1ST SACRAL
ANT. PRIM. DIVISION 5TH LUMBAR
SACRAL PLEXUS
PARTIALLY HERNIATED NUCLEUS PULPOSUS OF 5TH LUMBAR INTERVERTEBRAL DISC
ANNULUS FIBROSUS OF DISC
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Fig. 1 Cross-section anatomy at the level of the 5th lumbar disc to show relation of the first sacral nerve root to herniation of the 5th lumbar nucleus pulposus.

by surgery. Verification has been done by demonstration of single nerve root compression at operation, by traction on the exposed nerve root, by section of the nerve root in a small number of cases and by x-ray controlled novocaine injection of a single nerve root in medical student volunteers and clinical cases. Published recognition of this work has

been given by Reese ('44); Sachs ('45); Cerny ('47); Falconer, Glasgow and Cole ('47); White ('47); Bailey ('48), and by many others in personal communication.

The current anatomical concepts of segmental cutaneous nerve distribution are derived chiefly from Sherrington's

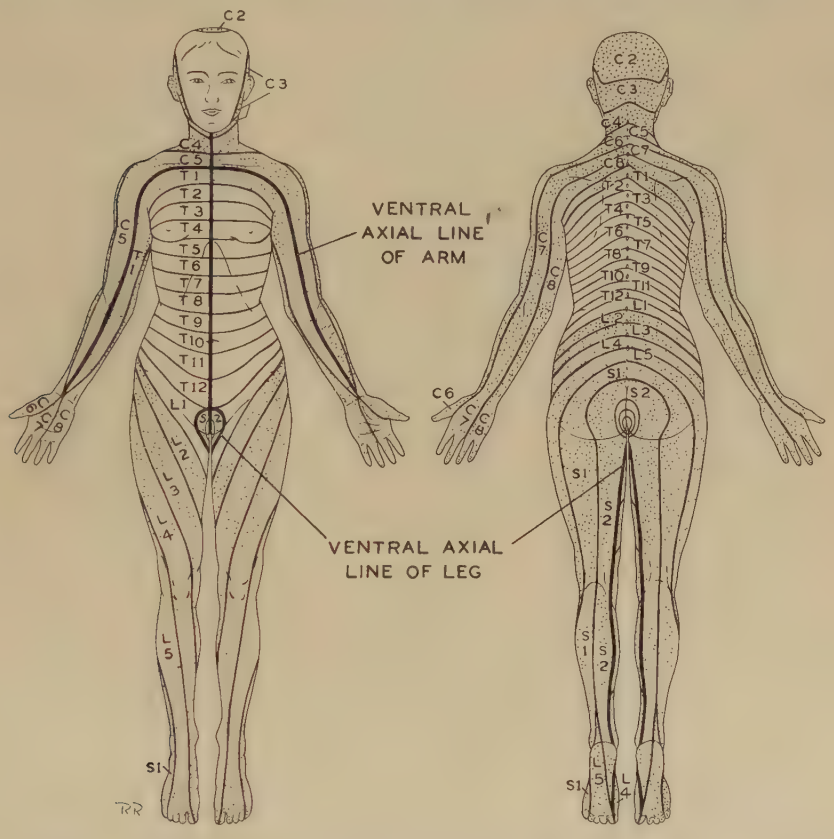

Fig. 2 Dermatome chart of the human body with new patterns in the extremities continuing in serial sequence from the spine.

(1892, 1893, 1898) physiological observations on monkeys, Bolk's (1898–1899) dissections of a human subject, and the careful clinical observations of Head (1893, '00) and of Foerster ('33, '36), supplemented by the experimental studies of Winkler ('03) and his pupils, and by a great mass of less

systematic clinical observations by various authors. The resulting outline of sensory dermatomes, while reasonably clear in the trunk, is imperfect and confusing in the extremities (figs. 3 and 4). Many authors, protesting against the prevailing confusion, have undertaken to draw a somewhat hypothetical dermatome chart which would illustrate in better

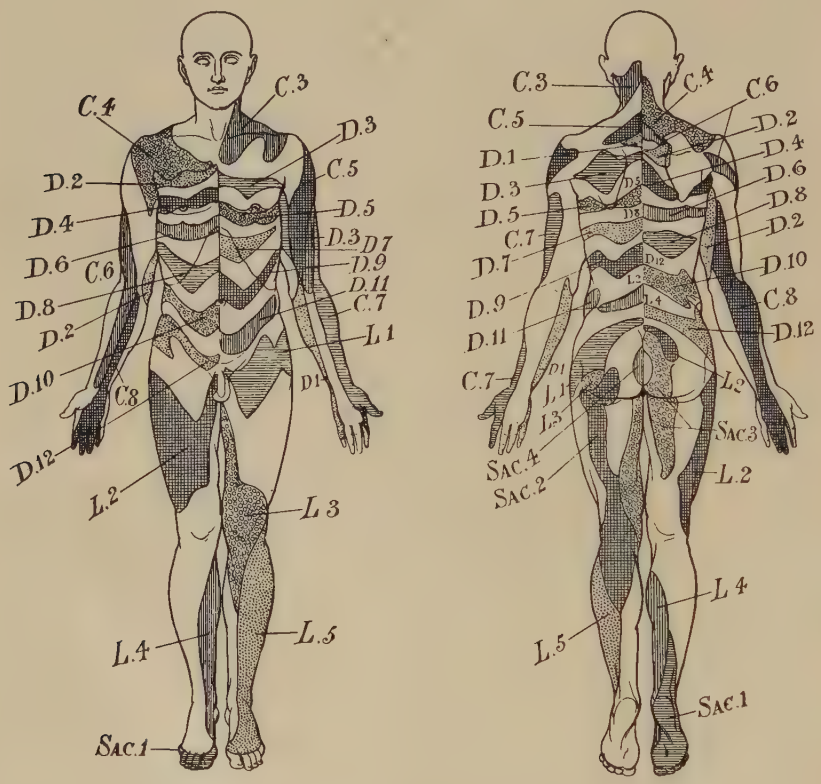

Fig. 3 Head's (1893) chart of the human dermatomes.

manner the serial segmental distribution of the dermatomes in the limbs. The most noteworthy early chart of this kind was prepared by Edinger ('04, fig. 5) and was used by Bing ('21). It is interesting to note the surprising agreement of Edinger's chart with that found in this study by plottong the area of decreased skin sensitivity for a single nerve root. A more

complicated early dermatome chart was drawn by Déjerine ('14) and has been used by Tilney and Riley ('21). While it is appreciated that long accepted findings of honored investigators as Sherrington, Head and Foerster should not be lightly questioned, yet it should be recognized that there is

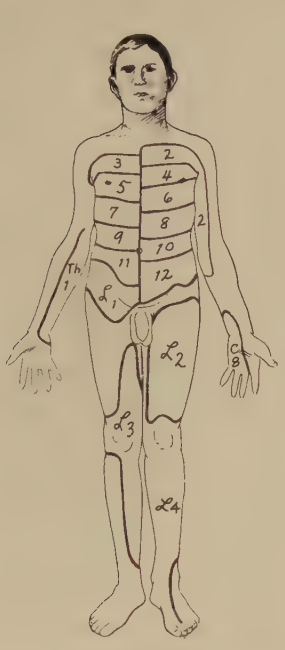

Figure 4

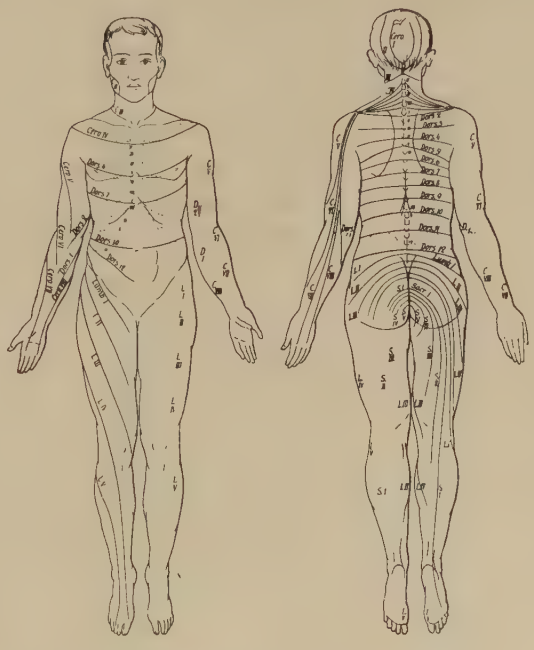

Figure 5

Fig. 4 Foerster's ('36) chart of the human dermatomes.
Fig. 5 Edinger's ('04) chart of the dermatomes.

possibility of incompleteness or error in their findings and interpretations, and that contradictory findings based on new technique and adequate material should be given careful consideration.

METHODS AND RESULTS

The relations of the nerve roots distributed to the extremities, as they leave the spinal cord, are particularly favorable for single nerve root compression by herniation of the nucleus pulposus of an intervertebral disc. In the lower lumbar

region (fig. 6) the nerve roots pass out obliquely, so that the common posterolateral nucleus pulposus herniation compresses a single nerve root, the one next below the numbered intervertebral disc. Thus herniation of the 5th lumbar disc compresses the first sacral nerve root and gives rise to radiating pain, first in the distribution of the posterior primary division to the gluteal region and later in the distribution of the anterior primary division down the posterior

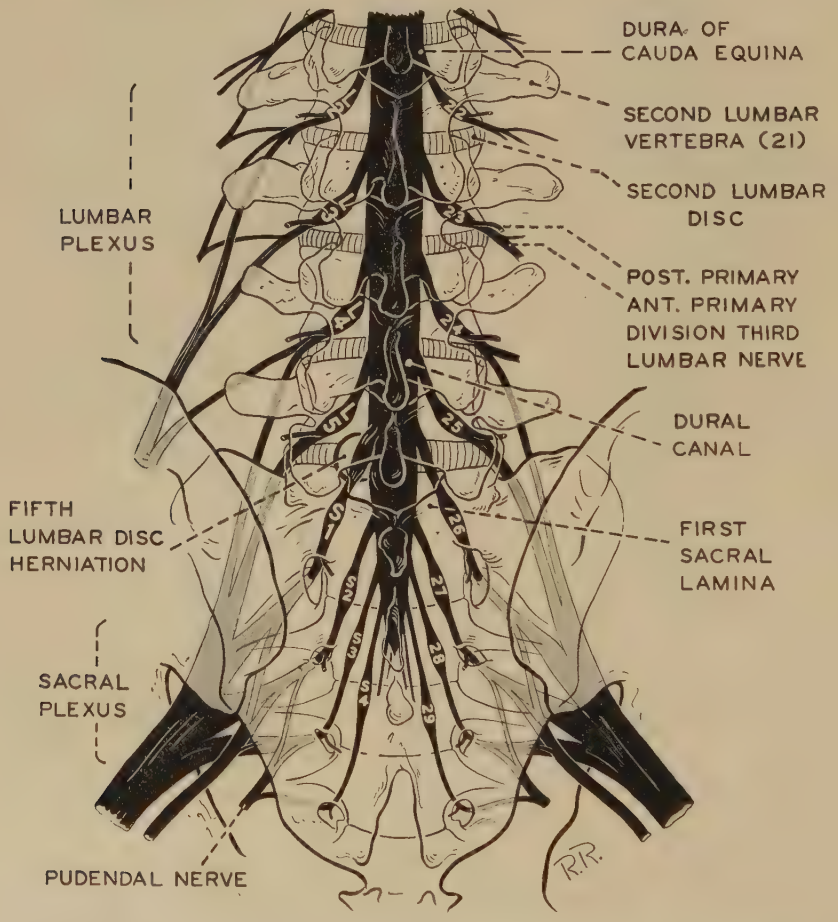

Fig. 6 Posterior view of the nerve roots of the lumbar and sciatic plexuses in relation to vertebrae and intervertebral discs.

surface of the thigh and calf, with corresponding sensory reduction (fig. 7). In the lower cervical region (fig. 8) the nerve roots pass out transversely so that posterolateral herniation of a disc there compresses the nerve root of the corresponding interspace, but not numbered the same, due to lack of a disc between the first and second cervical vertebrae and identification of the first cervical nerve root above the first cervical vertebra. Radiating pain first appears in the upper medial scapular distribution of the posterior primary

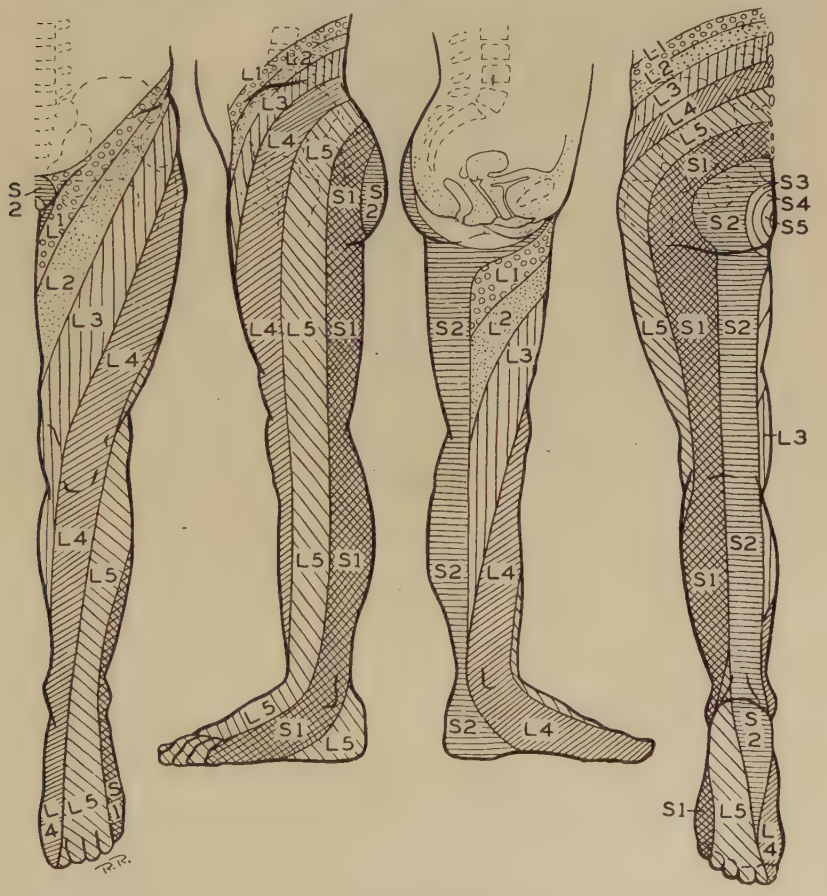

Fig. 7 Dermatome chart of the lower extremity of man outlined by the pattern of hyposensitivity from loss of function of a single nerve root.

division and later radiates over the shoulder and down the arm in fairly well localized dermatome distribution, with corresponding sensory reduction (fig. 9). During operation for removal of this discrete nucleus pulposus herniation it is necessary to retract the nerve root to expose the herniation tumor. This traction of a single nerve root, which often has

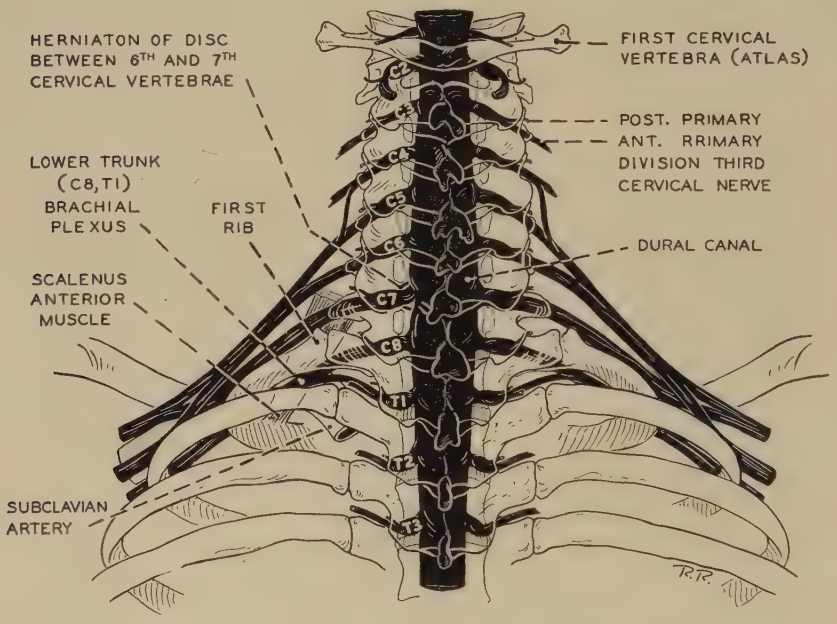

Fig. 8 Posterior view of the nerve roots of the brachial plexus in relation to vertebrae and intervertebral discs.

not completely lost its function before operation, leads to temporary complete loss of function of a certainly identified nerve root for postoperative testing. Added identification of nerve root loss has been found after purposeful section of a single nerve root to the lower extremity in 13 clinical cases and by novocaine injection of a single lower cervical nerve root in 10 medical student volunteers.[1] In the latter the

[1] Medical students injected: L. H. Blattspieler, R. Chesnut, E. Davis, R. Geesaman, T. Hubbard, J. Laws, W. McQuillan, L. Morgenstern, H. Rodman, V. Worthing.

desired position of the needle was secured by inserting it from an anterolateral direction until it contacted the transverse process of a cervical vertebra where the nerve root is closely applied as it leaves the intervertebral canal. With the needle in situ, roentgenograms were taken to establish its position and 2 cm of 2% novocaine then were injected around

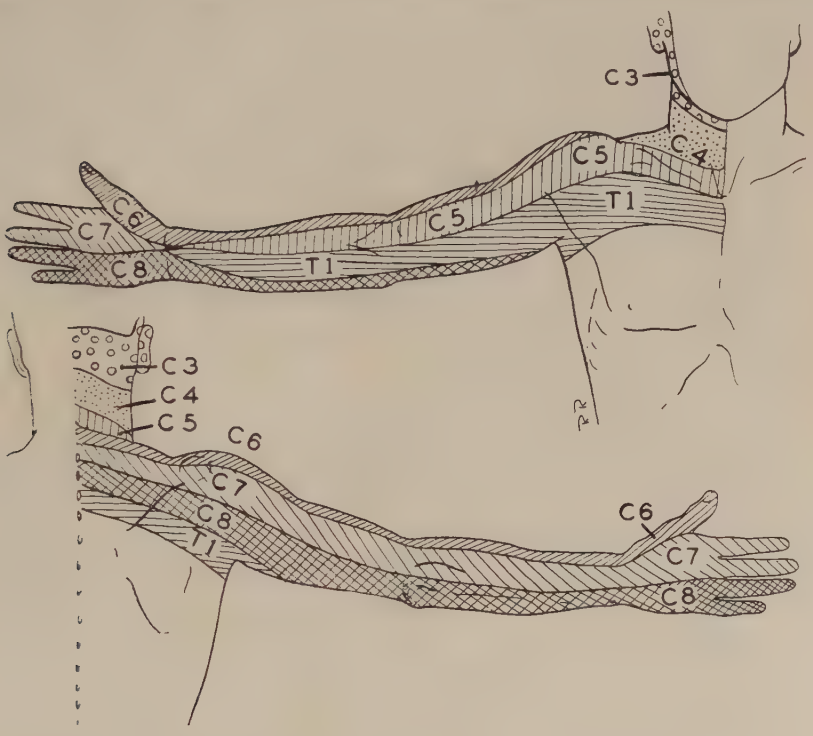

Fig. 9 Dermatome chart of the upper extremity of man outlined by the pattern of hyposensitivity from loss of function of a single nerve root.

the nerve root and found sufficient to block the sensory fibers of that root without spreading to adjoining roots.

The ability of the average individual to detect a diminution of sensitivity when function of a single nerve root is interrupted is contrary to the frequently voiced opinion that "division of a single nerve root produces no loss of sensitivity" (Foerster, '33). This opinion cannot be accepted,

for many patients with compression, traction or section of a single nerve root to an extremity are aware and even complain of an area of "numbness" which they locate accurately in the dermatome area in which faint but definite reduction of sensation can be demonstrated. The best method of marking out this area of detectable hyposensitivity is by use of light pin scratch for pain sensation, although it can be found for temperature and for tactile sensation also. The pain stimulus strength must be adjusted carefully so that it is not disagreeably painful in the area of diminished sensitivity; then the contrast with an adjoining more sensitive area becomes evident as the pin crosses into the more normal sensitive zone of the adjoining nerve root, often to the extent of surprised withdrawal. Experience enables one quickly to determine the proper pressure for a given individual. Testing is done entirely by free hand, using a medium size safety pin opened to right angle for better hand control and lightly scratching the skin from the hyposensitive zone across into the sensitive zone. A series of points on the line between hyposensitive and sensitive areas are marked with wax pencil and these points then are connected to outline an area of primary diminished sensitivity. The accuracy of the test which can be gauged by repetition does not vary more than one centimeter in well defined cases and the dermatome areas thus plotted do not seem to overlap significantly in different individuals. Sometimes, by continuing the pin scratch around the extremity, a secondary zone of very slight hyposensitivity can be found, representing the recognized overlap distribution of the nerve root. This is shown by photograph of a verified clinical case of left 6th cervical nerve root compression (fig. 10) right 5th lumbar nerve root (fig. 11) and right first sacral nerve root (fig. 12).

Since the dermatomes of the trunk are fairly well established and do not involve the question of limb bud migration, this investigation has been limited to those nerve roots sup-

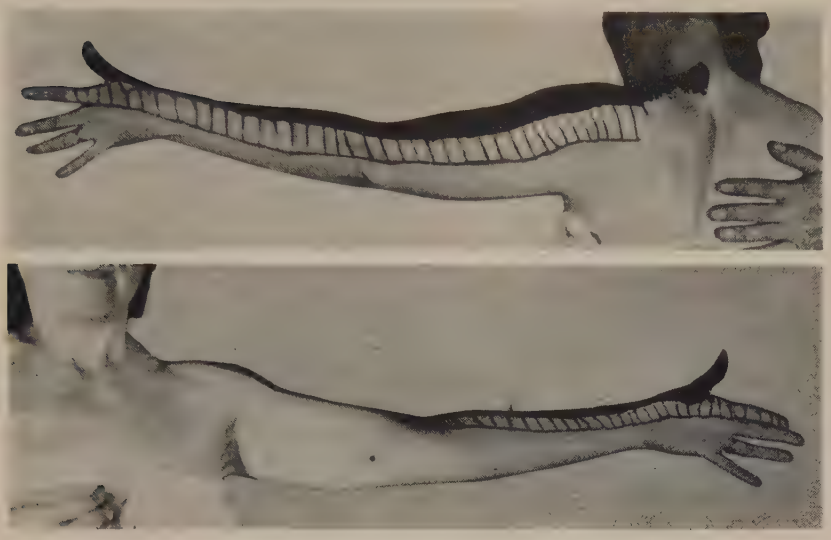

Fig. 10 Dermatome hypalgesia of 6th cervical nerve root with fainter overlap distribution.

Fig. 11 Dermatome hypalgesia of 5th lumbar nerve root with fainter overlap distribution.

plying the extremities, from the 5th cervical to the first thoracic and from the third lumbar to the second sacral. Thus the dermatome hypalgesia pattern has been plotted in 19 cases for the 5th cervical nerve root, 28 cases for the 6th cervical, 49 cases for the 7th cervical, 46 cases for the 8th cervical plus 53 cases of combined anterior primary divisions of 8th cervical and first thoracic nerve roots, 19 cases of third

Fig. 12 Dermatome hypalgesia of first sacral nerve root with fainter overlap distribution.

lumbar nerve root, 117 cases of 4th lumbar, 517 cases of 5th lumbar, 670 cases of first sacral and 19 cases of second sacral nerve root. This makes a total of 1556 plottings of a single nerve root dermatome hypalgesia, 826 of which were verified by surgery or x-ray. Additional cases of upper cervical dermatome hypalgesia have been found and will be reported in a later article by Dr. Alister I. Finlayson.

DISCUSSION

The exact anatomical significance of these dermatome areas of primary hyposensitivity from single nerve root loss is somewhat uncertain. They obviously do not represent the full area of cutaneous distribution of nerve fibers entering over a single nerve root, for the classical researches of Sherrington (1892, 1893, 1898) and Foerster ('33, '36) have shown that the total area supplied by each nerve root overlaps broadly with its neighbors on each side. Klessens ('13), by strychninizing individual nerve roots and plotting the area of hypersensitivity thus produced, was able to show as many as 4 different root distributions overlapping at some places. There must be, however, a definite difference of distribution between the area of primary hyposensitivity outlined in this study and the areas of overlap on each side, indicating that many more fibers of the root involved supply the primary area than the neighboring areas of overlap. This is supported by Head's (1893) finding of little overlap of dermatomes in his herpes zoster cases. Also there must be a relatively narrow zone of transition from the primary area of more dense distribution to the area of less dense distribution of the terminals, to account for the precision with which the hyposensitive area from single nerve root loss can be outlined. One might postulate a developmental sequence involving a primitive and primary connection of the specific root fibers with the hyposensitive area, followed by a spreading over the surrounding territory of overlap and establishment of fewer or less sensitive terminations in the secondary overlap area. This would imply that the hyposensitive area represents the true primitive dermatome, interpreted as that area of the skin most directly associated with the body segment in question through early contact with such segmental structures as blood vessels and nerves.[2] By contrast, the total area of distribution of nerve roots demonstrated by classical in-

[2] The authors believe it unlikely that the so-called dermatomic portion of the primitive somite retains any individuality in the developmental stages under consideration.

vestigators, and by them designated "dermatomes," would appear to have a less fundamental morphological significance than the primary hyposensitive areas of this study.

Whether or not the hyposensitive areas represent true primitive dermatomes, they must indicate rather accurately the

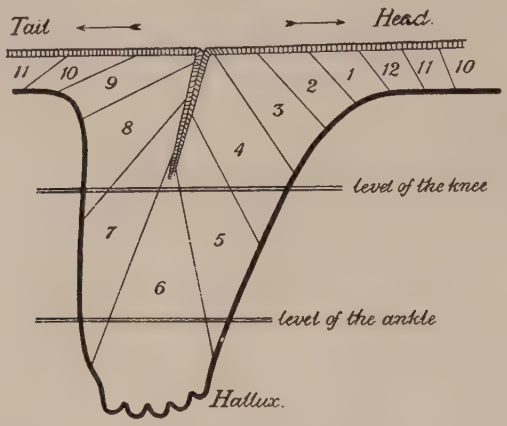

Fig. 13 Sherrington's (1893) "Scheme of the sensory skin fields of the hind limb of Macacus rhesus."

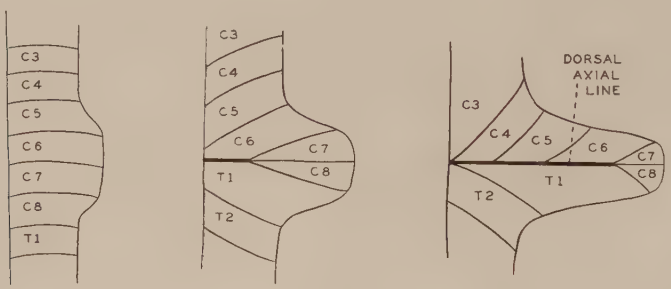

Fig. 14 Bolk's (1898) conception of the migration of the extremity dermatomes in limb bud development.

movements of the nerve fibers in the developing limbs. If so, they contradict the classical theories of dermatomic development in the limbs which are implied in Sherrington's (1893, 1898) description (fig. 13) more precisely formulated by Bolk (1898, II), (fig. 14). These theories postulate a simple out-

ward bulging of already delimited trunk dermatomes which are carried in the form of loops over the expanding limb buds, presenting dorsal and ventral [3] portions on the two surfaces of the limbs. These authors further postulate a separation of the limb dermatomes from the midline so that the dermatomes extend only to the dorsal and ventral axial lines of the limb, called "mid-dorsal" and "mid-ventral" lines of the limbs by Sherrington and "Differenzirungsgrenze" by Bolk. The results of this study indicate that dorsal axial lines of dermatomic junction have no reality; that the dermatomes continue unbroken from dorsal midline to their termination in the limb, and that they have developed in a very different manner than postulated in the loop theory. Reference again should be made to figure 2 which suggests the course of nerve development as a regular outgrowth over the dorsal surfaces of the limbs, assuming that the definitive position of the dermatomes indicates the course taken by the outgrowing nerve fibers. The highest and the lowest segmental nerves apparently have spiraled in a regular manner about the rostral and caudal limb borders respectively, to supply the ventral surfaces of the limbs and to meet each other along what might be termed the "ventral axial line." The intermediate segments have continued outward along the dorsal surfaces of the extremities to terminate near their tips. Reference to the extremity charts of this study adds clarifying details. Notice that in the arm (fig. 9), the highest and the lowest primary dermatomes, 5th cervical and first thoracic, spiral in opposite directions closely about the shoulder to meet in the axilla and run a parallel course along the ventral surface to terminate at the wrist. The remaining space around the arm is occupied in regular manner by the 6th, 7th and 8th

[3] Throughout this paper the usual nomenclature of limb surfaces is used as follows: *Dorsal*, the back of the hand and arm and convexity of the elbow; the top of the foot, front of the leg and convexity of the knee. *Ventral*, the palm of the hand, inside of the arm and concavity of the elbow; the sole of the foot, back of the leg and concavity of the knee. *Rostral*, the thumb and radial surface of the arm; the great toe and medial surface of the leg. *Caudal*, the little finger and ulnar surface of the arm; the little toe and lateral surface of the leg.

cervical dermatomes, covering the rostral, dorsal and caudal surfaces of the arm and all surfaces of the hand. The pattern on the leg is similar though somewhat less regular (fig. 7). There the first lumbar and second sacral spiral about the hip to meet on the medial side of the ventral surface, but the first

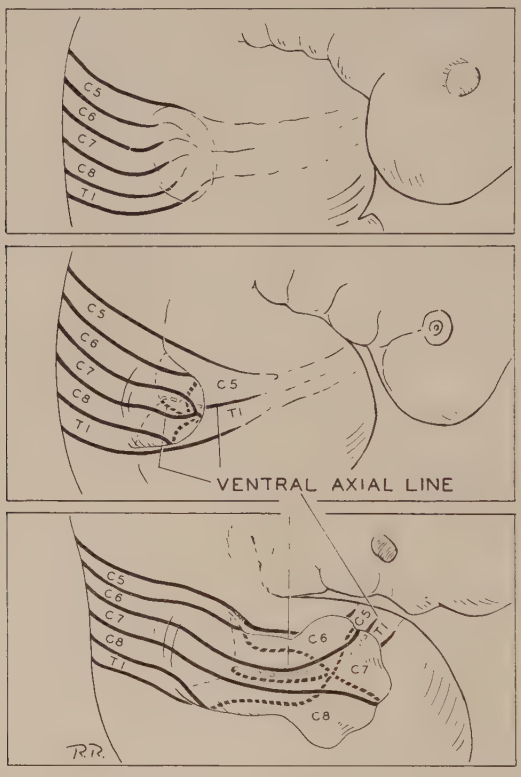

Fig. 15 The authors' conception of the development of the dermatomes in the limb bud.

lumbar ends here and only the second sacral continues down the leg. The second and third lumbar dermatomes likewise end against the second sacral and only the 4th and 5th lumbar and first sacral continue parallel with the second sacral to the end of the extremity where all interdigitate on the sole of the foot. An attempt has been made in figure 15 to show

this conception of the developing dermatomes in the limb bud of the human embryo at three different stages.

Since this interpretation of cutaneous limb innervation differs so sharply from that of Sherrington and of Bolk, and since none of the subsequent investigators have questioned their views, it has seemed advisable to compare in detail these dermatome findings with the dermatomes of the previous principal investigators, Sherrington, Bolk, Head and Foerster. This comparison is made by superimposing the areas from the charts of the above authors (diagonal lines) on corresponding areas of Keegan's chart for each dermatome (stippled) (figs. 18 to 27).

Sherrington obtained his results by isolating a single nerve root in the Macacus rhesus monkey, cutting two or three nerve roots above and below and determining the area of remaining pain sensation to skin pinch, registered by minimal motor reflex action. He thus obtained what approached the total distribution of the nerve root, showing considerable overlap with neighboring areas. The dermatome areas of the arm of the monkey are comparable to those of man and are transposed without modification. It is very difficult to compare the dermatome areas of the lower extremity of the monkey with those of man, both because of different conformation of the extremity and the question of analagous nerve roots. Sherrington (1892), from motor root stimulation experiments, interpreted the second lumbar nerve root of the Macacus analagous to the first lumbar root of man, and the roots below correspondingly. This is supported by the fairly constant abolition of the knee jerk by section of the sensory fibers of the 5th lumbar nerve root of the monkey (Sherrington, 1893), whereas in man the knee jerk most commonly goes out with the 4th lumbar nerve root loss. However, the closure of the vagina by the muscles of the pelvic floor of the monkey results chiefly on excitation of the 9th subthoracic nerve root, third sacral or 28th of the total series. This corresponds to the similar innervation of the pelvic floor of man by the 28th nerve root, 9th subthoracic, 4th sacral

or pudendal nerve. The anatomical similarity of the Macacus spine and nerve roots to those of man (fig. 16) has been called to attention (Keegan, '47b). Each has 7 cervical, 12 thoracic, and 10 subthoracic vertebrae to the end of the sacrum, the last or 28th nerve root of the total series contributing similarly to the pudendal nerve. From this it would seem that the only

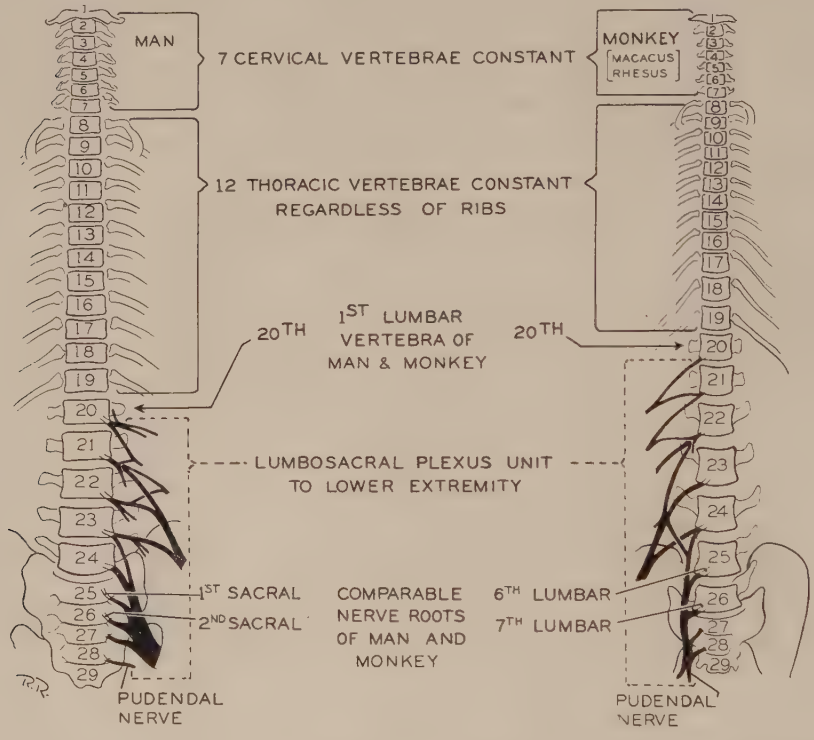

Fig. 16 Comparison of the spine and lumbosacral nerve roots of Macacus rhesus monkey and man.

significant difference beween the Macacus monkey's spine and man's spine is the fusion of the 6th and 7th lumbar vertebrae of the monkey into the sacrum of man. Also it has been found (Keegan, '47b) that nerve roots in man do not change in their cutaneous distribution with added or reduced lumbar vertebrae, if the vertebrae are identified by total numerical sequence and not by variable number of ribs or lumbar ver-

tebrae. Accordingly, in this study, comparison of Sherrington's chart of the dermatome areas in the lower extremity of the monkey with the dermatome hypalgesia areas has been made by considering the first 5 lumbar nerve roots of monkey and man analagous, the 6th and 7th lumbar nerve roots of the monkey analagous to the first and second sacral nerve roots of man, and the first and second sacral nerve roots of the monkey analagous to the third and 4th sacral roots of man.

Bolk's data resulted from careful gross dissection of a single human body and tended to present the total distribution of a nerve root, but unavoidably lack the distribution of the fine branches. His charts are used without modification.

Head (1893, '00) drew his data largely from the distribution of skin lesions in cases of herpes zoster involving a single nerve root, but obtained a very irregular and incomplete picture in the extremities, with little or no overlap (fig. 3). He reported no examples of isolated 8th cervical or first sacral involvement in 392 clinical cases and verified only one case in the lower extremity by post-mortem examination, this of the first lumbar nerve root. This would leave some question concerning the accuracy of Head's dermatome areas in the extremities. His charts are used without modification. An interesting observation has been made on a clinical case of herpes zoster in the lower extremities, using light pin scratch technique to outline the area of hyperalgesia recognized to accompany these single nerve root herpetic lesions. It was found that a clearly defined dermatome area of hypersensitivity could be outlined (fig. 17), which included the herpetic eruption but was more extensive and corresponded accurately with the third lumbar and second sacral dermatome areas as previously reported in herniated disc cases by hyposensitivity finding. This indicates that the herpes zoster dermatome areas outlined by Head might have been amplified by outlining more accurately the associated areas of hypersensitivity, to give a dermatome chart corresponding to that of this study (fig. 2).

Fig. 17 Photograph of the areas of herpes zoster and associated dermatome pattern of hyperalgesia found in the lower extremities of a clinical case.

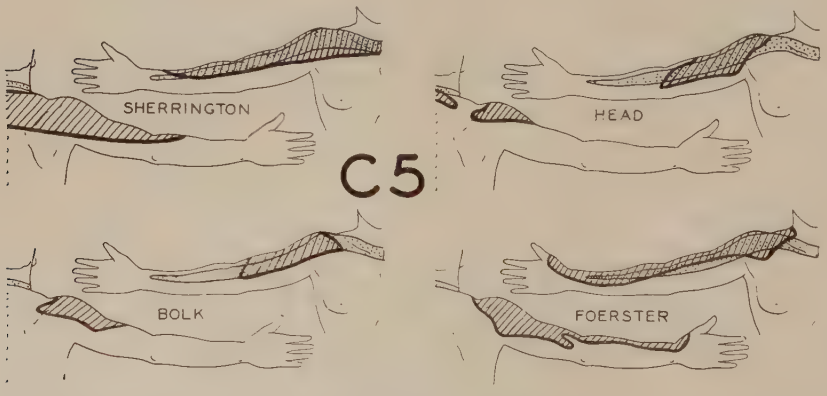

Fig. 18 Fifth cervical dermatome comparison. There is agreement in placement of this dermatome over the biceps swelling, with variable extension proximally and distally, but none to the digits. Evidently distinguishment between primary and overlap sensation has been difficult and confusing with other authors.

Foerster ('33) followed the same method as Sherrington in considerable degree, cutting nerve roots above and below an identified nerve root and plotting remaining tactile and pain sensitivity. He found the remaining tactile dermatome larger than the pain dermatome, with considerable overlap of dermatomes. He also used faradic stimulation of a single nerve root and plotted the resultant vasodilatation dermatome which was less extensive than the tactile and pain dermatomes, approaching the non-overlapping herpetic dermatomes of Head. These

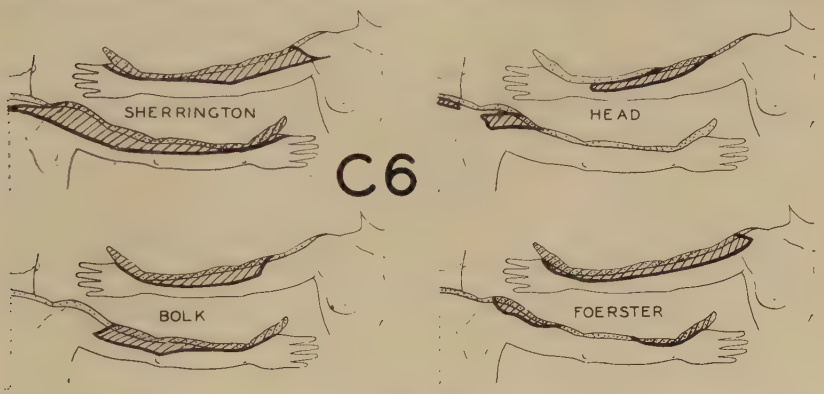

Fig. 19 Sixth cervical dermatome comparison. There is surprising agreement of location along the rostral or outer side of the arm, with variable extension towards the spine.

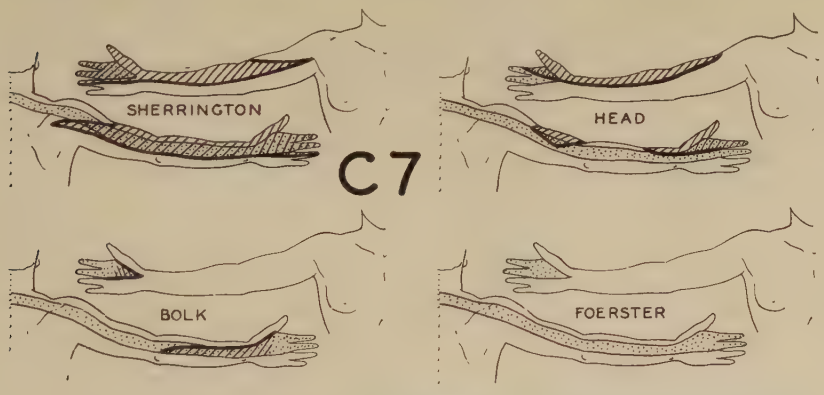

Fig. 20 Seventh cervical dermatome comparison. Distally there is fair agreement in inclusion of the index and middle fingers. Bolk's agreement distally is remarkable. Foerster did not show this dermatome.

variables, including considerable individual case variation, make comparison of Foerster's chart of the dermatomes (fig. 4) with others difficult. Insofar as possible the dermatomes he outlined for pain have been used in this study, taken from individual case findings.

The most conspicuous difference between our findings and those of the other authors lies in representation of complete dermatomes extending without interruption from the dorsal

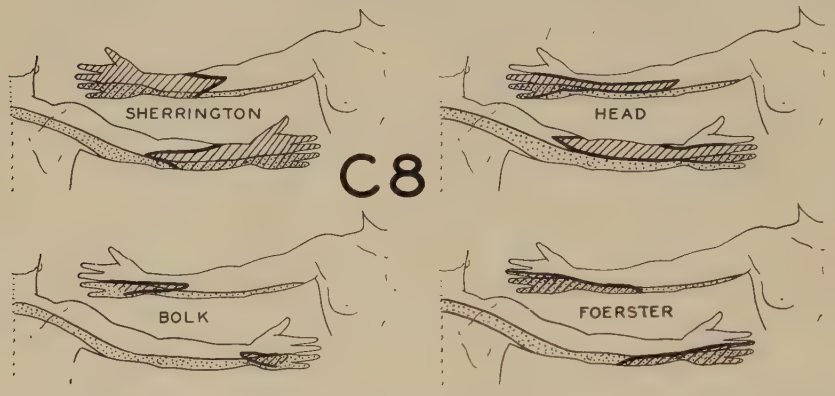

Fig. 21 Eighth cervical dermatome comparison. All charts agree in showing involvement of the ulnar portion of the hand, with Bolk's almost identical. Sherrington and Head show dorsal and ventral portions of a dermatomic loop but with considerable variance in location.

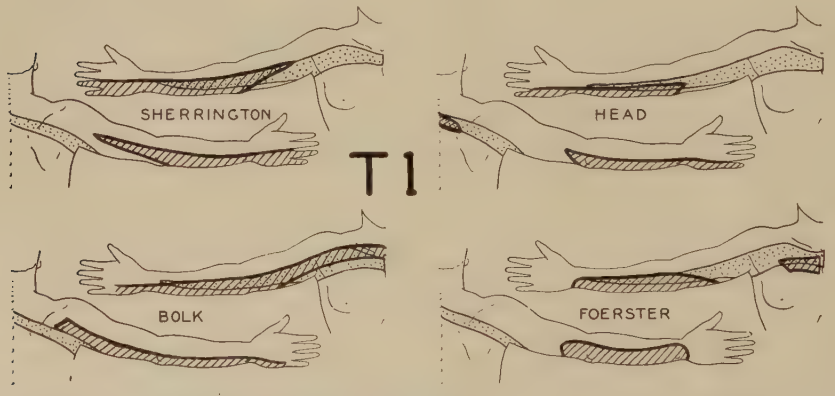

Fig. 22 First thoracic dermatome comparison. On the forearm, agreement between all 5 authors is reasonably close. Bolk and Foerster do not include the little finger.

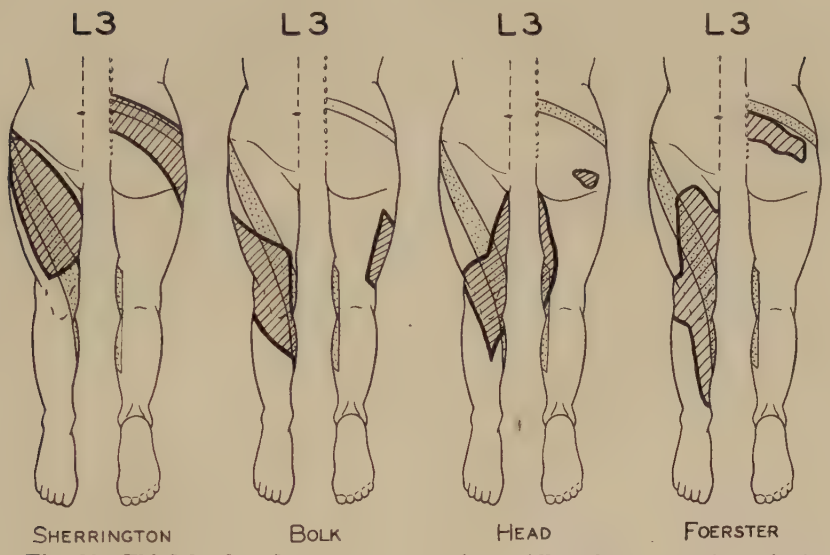

Fig. 23 Third lumbar dermatome comparison. All authors agree in assigning the territory on the medial side of the knee in man. The transposed third lumbar area of Sherrington agrees surprisingly well in the thigh except for its greater extent.

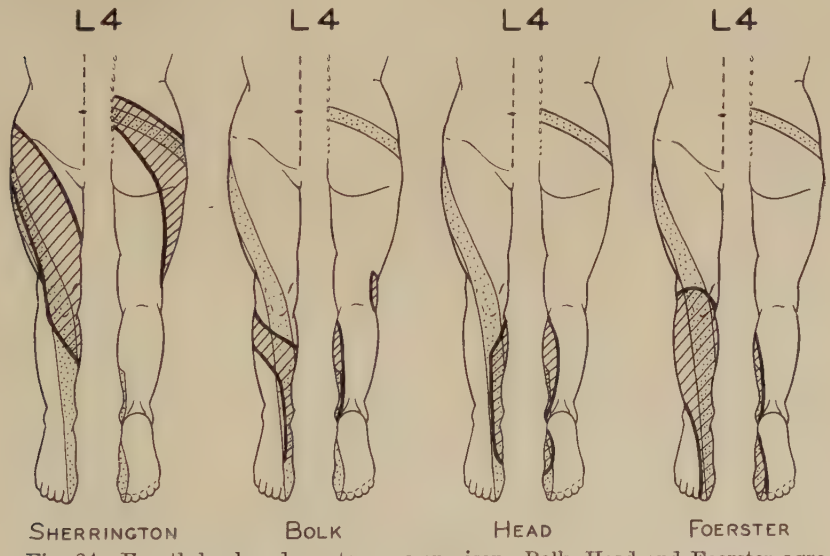

Fig. 24 Fourth lumbar dermatome comparison. Bolk, Head and Foerster agree in including the territory on the medial side of the ankle and foot. Sherrington's larger oblique area is in general agreement above the ankle.

midline to their termination, whereas the other authors frequently represent gaps as occurring in the region of the upper portions of the limb. Interesting in this connection are the variations in degree of hyposensitivity which is found within the area of diminished sensitivity. There often is a rather limited portion of the area which approaches complete analgesia to light pin scratch and may be noticed independently by the patient himself. This usually is located rather distally in the area except in the case of the second sacral nerve. Proximally the degree of sensory impairment tends to diminish so that in the gluteal or scapular regions considerable care is required to delimit the involved area. Clinical cases with incomplete pressure block of the nerve root may show hyposensitivity only in the more distal portion of the limb. It should be emphasized that the location of the line of dermatome junction between primary hyposensitive and adjoining areas is uninfluenced by these changes of upper level of hyposensitivity and is found in the same position as long as it can be demonstrated. It is on this failure to demonstrate the more proximal portions of the dermatomes in the extremities that Sherrington and Bolk principally based their theory of the migration of these dermatomes and the significance of axial lines.

It appears that the only suggestion of a confirmation of Sherrington's theory of dermatomic loop configuration brought out by our comparisons is the representation of the 8th cervical by Head showing what appear to be dorsal and ventral portions of a loop. Even here the correspondence with Sherrington's area is not close. It seems likely that through some chance the ulnar border escaped involvement in the herpetic lesion which formed the basis of Head's description, and that, therefore, the loop configuration is purely accidental. It further appears that in no case is there a fundamental difference between Sherrington and the other investigators in locating the main mass of an area; that Sherrington's loop interpretation depends on relatively slight extensions, all without confirmation, from the territory indicated by the

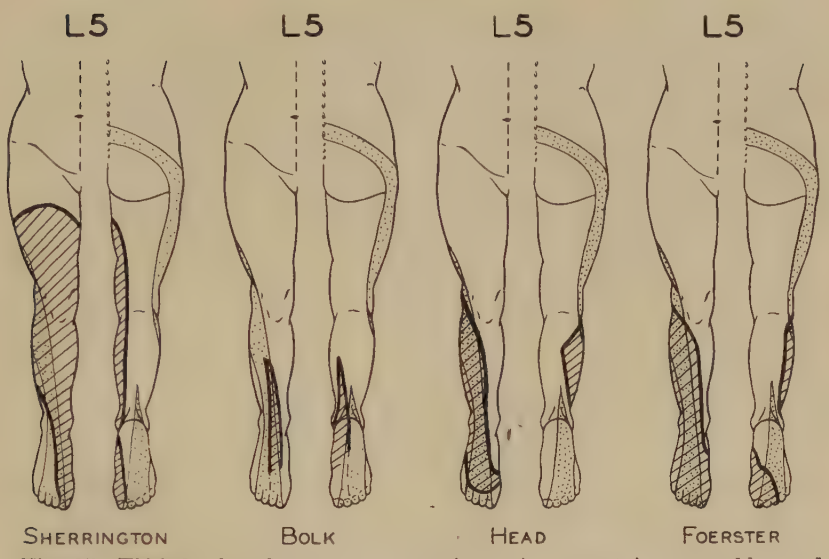

Fig. 25 Fifth lumbar dermatome comparison. Agreement is reasonably good. The transposed 5th lumbar area of Sherrington is more medially located.

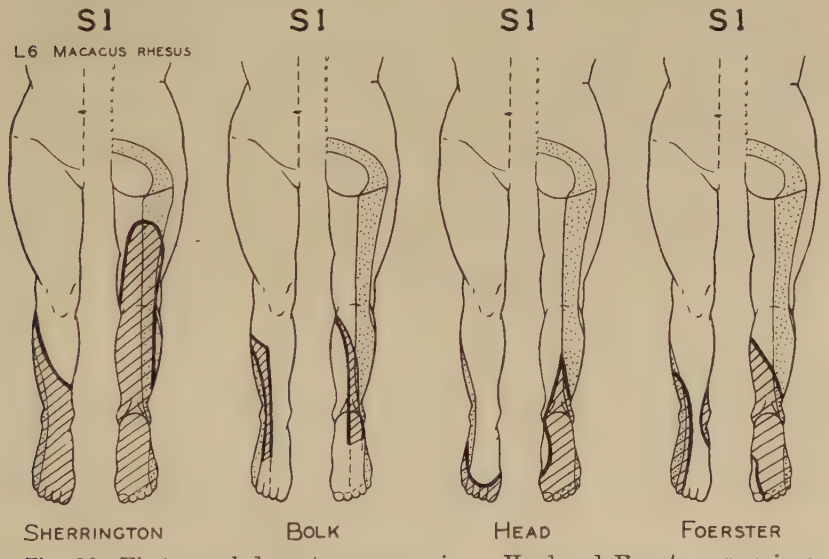

Fig. 26 First sacral dermatome comparison. Head and Foerster agree in assigning this area to the lateral side of the foot and small toe and in excluding all or a portion of the medial side of the foot. Sherrington's 6th lumbar area of the monkey includes all of the foot and posteriorly in the calf and thigh.

other charts. We feel the conclusion justified that Sherrington erred through a relatively minor, though systematic, misinterpretation of his data, and that "dermatomic loops" and "dorsal axial lines" do not exist. The concept of a ventral axial line may be retained for the line of junction between more rostral and more caudal dermatomes after they have spiraled about their respective borders of the limb. This criticism of Sherrington can be applied with even greater

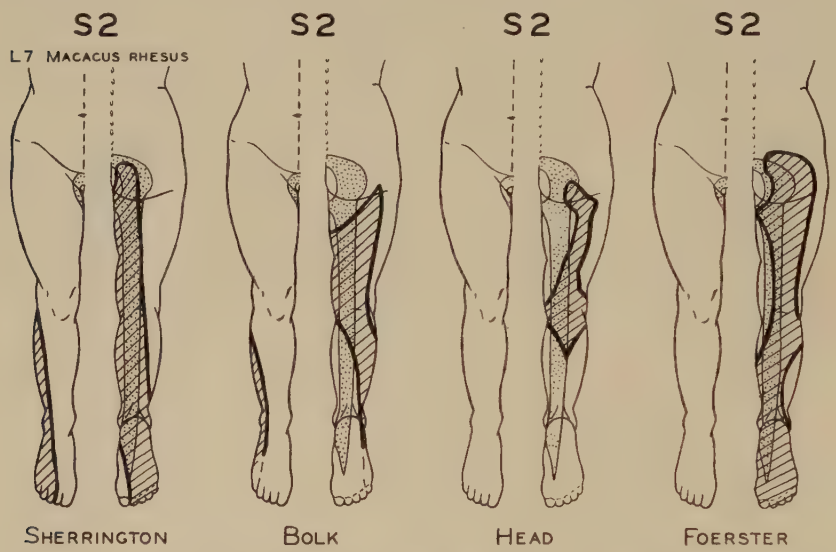

Fig. 27 Second sacral dermatome comparison. All authors agree on a distribution to the medial side of the back of the knee, this being in surprising agreement with Sherrington's 7th lumbar of the monkey. There is quite variable extension into the sole of the foot.

certainty to Bolk. His charts differ chiefly in being less complete. When one considers his method, gross dissection, it becomes remarkable that his results are so similar.

It has been established (Weiss, '41) that the course of a nerve fiber in the definitive organism is determined, first, by the pathway taken by the freely moving tip of the fiber prior to contact with its end organ and later by the towing of the fiber after it becomes attached to the end organ or skin. On the basis of this doctrine the loop hypothesis of Sherrington

and of Bolk would imply that the fibers had attained peripheral attachment to the skin organs prior to initiation of limb development, for dermatomic loop would result only from towing of attached fibers as the skin bulges outward to cover a forming limb bud. Bolk based his hypothesis of dermatome development on the now largely discredited fin fold theory of limb development and thus actually described the dermatome as bending over the margin of a horizontal ridge rather than bulging over a limb bud. Dermatome development as we postulate it could occur, obviously, only in connection with a limb bud. Our interpretation, on the contrary, implies a free course of outgrowing fibers through the substance of an already formed bud (fig. 15). The actual course of early innervation of the limb has recently been described in the frog by Taylor ('43). He has determined that the very early limb bud is, in fact, nerveless; that the first fibers grow freely into the limb substance guided, apparently, by the orientation of mesenchymal strands. In a later paper Taylor ('44) shows that some as yet unknown factor determines connection of sensory (and probably autonomic) fibers with the skin and motor fibers with the muscles. Our observations seem to imply that these guiding agencies, whatever they are, must be rather general in their effect, for the primary factor determining the exact attachment of a given fiber has obviously been the even and orderly filling of the space to be occupied.

CONCLUSIONS

A cutaneous area of detectable hyposensitivity results from blocking a single spinal nerve supplying a limb. These areas extend as continuous bands or dermatomes from the spine into the limb, contrary to the theory of the loop development of the dermatome. These newly defined dermatomes of non-overlapping primary hyposensitivity are interpreted as the primitive dermatomes.

This study indicates that postulates of a loop configuration of the dermatomes and of dorsal axial line of dermatomic juncture are untenable. The sensory nerve fibers grow spirally

from the dorsal surface of the limb bud, around both rostral and caudal borders, to meet on the ventral surface along a ventral axial line.[4]

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A HERNIA THROUGH THE MUSCULAR LACUNA IN A NEWBORN MALE PUPPY

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ONE FIGURE

Hernias occur in man most frequently in the inguinal region, while in the 4-footed animals, hernia is reported most frequently in the umbilical region or through the ventral abdominal wall. Diaphragmatic hernia is found both in man and in animals. Kent ('45) has reviewed 4 cases of diaphragmatic hernia in the cat and then describes one new case which he found. Phillips and Felton ('39) have observed umbilical hernia in collies, bull terriers and cocker spaniels. They have observed cases but have no records of the number of cases in the first two breeds of dogs, but for the cocker spaniels, they say that over a period of 20 years of breeding cocker spaniels they have observed 10 female and 8 male dogs with umbilical hernia. They also add, "in this kennel no other forms of hernia were ever observed." Warren and Atkeson ('31) report studies on umbilical hernia in Holstein-Friesian cattle. They feel that hernia of this type is inherited in a simple Mendelian fashion.

Brumley ('38) describes both inguinal and femoral hernia in the small domestic animals. Inguinal hernia he says, "is not common although it is met with occasionally." Concerning femoral hernia, he says, "This form of hernia is not common in small animals." He describes inguinal hernia as passing through the inguinal canal and femoral hernia as passing through the femoral canal just as they do in man, and with similar relations.

There are relatively few cases of hernia reported for the smaller domestic animals, but without doubt many more do occur than are described in the literature. This case is unlike any that have been described, so far as our search of the literature shows, in either man or any of the animals and it is so unusual that a brief description of it seems justified.

The male puppy with this hernia was one of a series of 140 fetal and newborn puppies which is being studied to determine the growth of the various organs. Seven anomalies were found in the first 79 specimens (Latimer and Corder, '46). Additional anomalies have been found in the last 61 specimens, including this hernia which will be described. In addition to this one there were two other hernias. An umbilical hernia was found in a 7-gm male fetus with about 2 cm of the small intestine protruding through the body wall. In a 262-gm female fetus the spleen had herniated through the lateral lumbocostal arch, carrying all of its vessels with it into the thoracic cavity.

This male newborn puppy was one of a litter of 4, born during the night, found by the service man and brought to the laboratory early in the morning. This litter of one male and three females seemed to be normal newborn puppies and they were only casually examined. Following the usual routine for all of these newborn puppies, they were chloroformed, then the thoracic cavities and the abdominal cavity were filled with a 10% solution of formalin, by means of a hypodermic syringe. An opening was made at the base of the skull so that the formalin could have easy access to the brain and spinal cord. Then they were immersed in an adequate amount of 10% formalin until removed for dissection. This male puppy had a body weight of 236.0 gm. He was heavier than the three females for two of them weighed 226.5 gm and the third, 188.0 gm. The body length (nose-anus) of this male was 191 mm and the lengths of the three females were 182, 196 and 190 mm.

The stomach of this male was well filled with coagulated milk and a little of it had entered the beginning of the small

intestine. The lacteals leading from the proximal end of the small intestine were very evident, due to the absorption of some of the fat from this milk. The entire length of the thoracic duct was filled as well. The three female puppies had no milk in their stomachs and of course no lacteals were evident. Does this mean that this male was the first one born and that he alone had had time to nurse? All 4 appeared to be normal healthy newborn puppies when they were brought into the laboratory in the morning. It is too bad that they were not examined more carefully so that the appearance of this hernia could have been described in the living.

Nothing unusual was expected and this puppy was dissected in the usual manner. The head was dissected first, then the thorax, and lastly the abdomen. Nothing unusual was noticed in the dissection of the head and the thorax, but when the dissection of the abdomen was begun the abdominal wall seemed softer or less tense to the touch than usual in the newborn puppies. Upon the removal of the skin of the abdomen and the proximal part of the thigh, this herniation was discovered.

Approximately one-third, or a little more, of the length of the small intestine had herniated under the inguinal ligament and was ballooned out under its coverings on the proximal, medial side of the right thigh. The herniating mass had followed the course of the psoas major muscle, or it had passed between the lateral part of the inguinal ligament (in fig. 1) and the superficial surface of the psoas muscle (P. in the figure). It passed out lateral to the abdominal inguinal ring, the iliopectineal ligament, the inferior epigastric artery, the pectineus muscle, which in the dog arises in part from the medial end of the inguinal ligament, and it was lateral and superficial to the femoral vessels (F). The structures deep to the surface are not shown in the accompanying drawing which shows only the superficial view of the abdominal wall after the removal of the skin and superficial fascia. The gubernaculum testes, which is a good-sized cord in these newborn puppies, seemed to have been pushed medially, and ex-

tending laterally from it and the abdominal inguinal ring were two folds of peritoneum, one above and one below the exit of the gubernaculum and extending for some distance laterally. These folds gave the appearance of a pushing of the ring medially and they represented tension lines in the peritoneum and the subjacent fascia. The testes in most of these newborn puppies had descended, or at least they had passed through the abdominal inguinal ring into the inguinal canal, but in this dog, the testes on both sides were about 4 mm from

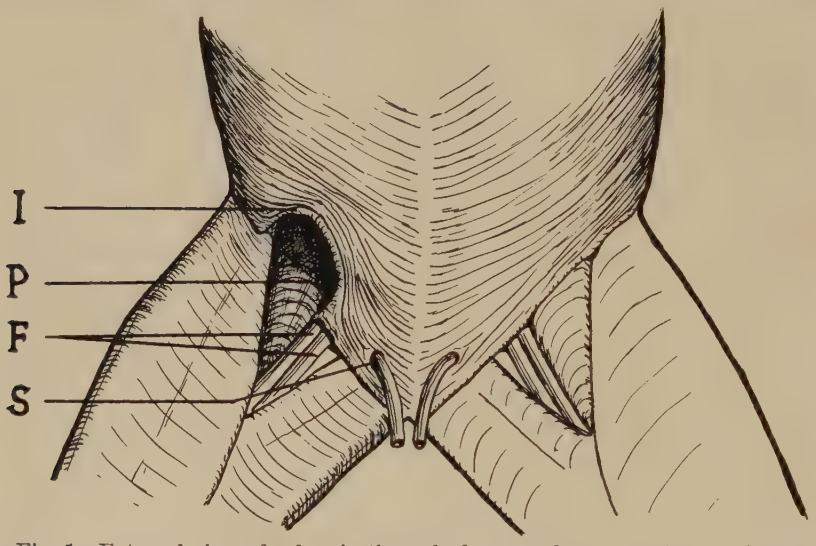

Fig. 1 External view of a hernia through the muscular compartment of a newborn male puppy. I, inguinal ligament; P, psoas major muscle; F, femoral artery and vein; S, subcutaneous inguinal ring with the gubernaculum testes.

the abdominal inguinal ring, with the gubernaculum extending out from each and passing through the canal into a normal processus vaginalis.

This opening in the muscular lacuna was a nearly symmetrical arch. The deep border was approximately straight and was formed by the muscular wall of the thigh and the anterior border of the ilium. The anterior wall which was the curved side of the opening, was made by the taut inguinal ligament. The opening was about 6 mm in height and 12 mm in

length. The course of the hernia as has been described, seemed to follow the course of the psoas major muscle as it passed under the inguinal ligament, and it did not go through the femoral canal and medial to the femoral vessels as a typical femoral hernia does. It was distinctly lateral to the femoral vessels as shown in figure 1 and passed through the muscular compartment rather than the vascular compartment. The right psoas muscle seemed to be perfectly normal and it was the same size and did not differ from the left psoas muscle in any way.

External hernias are usually named according to the region of the body wall through which they pass. For example: inguinal hernias pass through the inguinal canal; femoral hernias, through the femoral canal; umbilical, through the umbilicus, and so on. A shorter name for this type of hernia, and also in some ways a more descriptive name, would be a psoas hernia. The name psoas hernia is suggested because of its close relationship to the psoas muscle.

This herniated mass was deep to the skin and also to a fascial layer which seemed comparable to the fascia lata of human anatomy. This fascial layer was thin and seemed to be continuous with the fascia covering the abdominal wall, and it was but slightly attached to the inguinal ligament. Bradley ('43) says that "from the (external) oblique muscle a thin ill-defined sheet is reflected onto the medical aspect of the thigh." The layers covering the herniated mass, deep to this layer which probably was the fascia lata, were in order from outside inward, fascia transversalis, a very small amount of extraperitoneal fat and lastly the peritoneum. There was usually but very little extraperitoneal fat in puppies of this series.

The details of the fascias of this region have not been found very well described for the dog, but they do seem to resemble the similar layers of human anatomy and so the names used for the human fascial layers have been employed. This region was dissected in an adult dog and the weakness of the entire inguinal region was strikingly evident. Miller ('47) in a

study of the inguinal region in primates finds a true inguinal ligament present only in man and a study of this region in the dog shows that here it is even less well developed than in the primates.

After a careful search of the literature and a study of the anatomy of the inguinal region in the dog, the surprising thing is not that a case of hernia has been found through the muscular lacuna under the inguinal ligament, but that many similar cases have not been recorded in the literature.

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THE HISTOCHEMICAL LOCALIZATION OF ALKALINE PHOSPHATASE IN DEMINERALIZED BONES OF MICE OF DIFFERENT AGES

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FIFTEEN FIGURES

Since the introduction of the technique for the histochemical localization of alkaline phosphatase (Gomori, '39 and Takamatsu, '39), many studies have been made on the soft tissues of different vertebrate forms. The mineralized structures, on the other hand, have received but scant attention. This has been due chiefly to the fact that unless the hard structures are demineralized prior to incubation with the substrate solutions, it becomes extremely difficult to distinguish between the localization of the phosphate pre-existing in the sections and that laid down during the incubation as a result of enzymatic action. A number of early attempts were made to overcome the difficulties (Kabat and Furth, '41; Gomori, '43; Bourne, '43) but none proved entirely satisfactory. Recently Lorch ('46) has published the details of a procedure whereby complete demineralization of bone can be accomplished without complete inactivation of the enzyme. Subsequently Greep, Fischer, and Morse ('47) reported a similar technique and extended their observations to include teeth as well as bones.

As a result of the biochemical studies of Robison and his associates, it is now generally accepted that a relationship exists between alkaline phosphatase and the mineralization of bones. These and other authors who subsequently investigated developing bones by histochemical methods (reviewed by Gomori, '43 and Moog, '44) have been able to demonstrate that the enzyme which hydrolyzes monophosphoric esters is

always found in tissues undergoing active ossification. This enzyme becomes evident only after the appearance of a center of ossification and increases in amount with the progress of histological differentiation. Although the enzyme may be present in tissues which do not become mineralized, normal mineralization apparently does not occur in its absence.

A number of questions about bone phosphatase have not been conclusively answered. Important among these are the questions of the site of origin and activity of the enzyme within the bone and the exact mechanism whereby it exerts its effects. In 1924 Robison and Soames reported the interesting fact that the bones of rachitic rats contain a larger amount of the enzyme than do those of normal rats. This suggested the possibility that a study of the bones of animals which have been subjected to experimental conditions designed to alter the normal bone structure and metabolism might contribute information of value in an elucidation of the above-mentioned questions. The histochemical approach using demineralized material seemed desirable because it would provide qualitative data, i.e., detailed descriptions of the actual histological and cytological localizations of the enzyme coupled with rough quantitative data. Preliminary to the experimental study, however, the histochemical localization of the enzyme in a series of normal animals was investigated as a function of development, growth and aging. It is the purpose of this paper, therefore, to report the findings of a study of the localization of alkaline phosphatase in the long bones of mice of different ages from the 15th day of gestation to 13 months of age. Since the beginning of this work Lorch ('47) and Greep and Morse ('48) have reported studies on the localization of the glycerophosphatase in demineralized bones of kittens, adult mice and young and adult rats. The results of these two groups of authors are in close agreement with each other and with those to be reported here for mice of corresponding ages.

The author wishes to thank Dr. Barnet M. Levy for his helpful criticisms and kind assistance.

MATERIALS AND METHODS

All the mice used were of the Swiss strain and were bred and raised in the laboratory. In order to obtain embryonic stages paired matings were made in the late afternoon and the females were examined early the next morning for the presence of a vaginal mucous plug which was taken to be indicative of copulation. The age of the embryos was calculated using the time of finding the mucous plug as the starting point.

TABLE 1

AGE IN DAYS	NUMBER OF MICE	AGE IN DAYS	NUMBER OF MICE	SEX ♂	SEX ♀
Pre-natal [1]		Post-natal			
15	4	1	4	2	2
16	2	3	4	2	2
17	4	5	4	2	2
18	4	7	4	2	2
19	6	10	3	1	2
		15	4	2	2
		17	3	3	0
		21	2	0	2
		23	2	1	1
		35	2	1	1
		40	2	1	1
		59	2	1	1
		167	4	4	0
		245	2	2	0
		291	1	1	0
		319	2	2	0
		395	2	2	0

[1] Sex was not determined.

With the exception of the embryos all the animals used were killed by a blow on the head and were immediately autopsied. The right tibia was isolated, cleared of adhering muscle and connective tissue, and fixed in chilled 80% alcohol. The same procedure was followed for the left knee joint. In table 1 is a summary of the total numbers of animals used, the ages, and numbers of each sex.

The procedures used in fixation and demineralization were modified from those of Lorch ('46) and were as follows:

1. The bones were fixed for 24 hours in 80% alcohol at about 5°C.

2. After rapid hydration each bone was placed in an individual flask containing approximately 100 cm^3 of sodium acetate-acetic acid buffer at pH 4.55–4.58 and allowed to demineralize in the refrigerator. The buffer solution was changed daily and the progress of demineralization checked using the calcium oxalate test described by Morse ('45) and, in the case of the older bones, by taking x-ray pictures. Demineralization required from one to 22 days. (Embryonic and one-day post-natal bones were not demineralized.)

3. After demineralization the bones were rinsed in distilled water and placed in a 10% solution of sodium barbital at approximately pH 9.85 for one hour in order to reactivate the enzyme.

4. The tissues were dehydrated rapidly, cleared in benzene for one hour, and infiltrated with paraffin at 56°C.

5. Sections were cut at 6 μ and mounted using as little heat as possible to spread the ribbons.

Incubation of the sections was carried out according to the Gomori ('39) technique using a 3.2% solution of sodium glycerophosphate (Eastman Kodak no. 644) as substrate. The incubation times used were 15 minutes, one-half hour, one hour, one and one-half hours, and two hours. Sections adjacent to those used for incubation with substrate were incubated in control solutions lacking the substrate. Visualization of the precipitate was carried out by the ammonium sulfide method (Gomori, '39).

RESULTS

I. Pre-natal development

At 15 days of gestation the lower limb skeleton was entirely cartilaginous. Areas were present, however, in which

the processes leading towards bone formation had begun. Similar areas, called ossification centers, have been described in other vertebrates (Bloom and Bloom, '40). As development proceeded the histological appearance of the centers changed due to what may briefly be summarized as: enlargement of the lacunae with decrease in cartilage matrix; vesiculation of the nuclei; vacuolization of the cytoplasm; increase in irregularity of cell outlines; and decrease in stainability. It was interesting that in the lower limb skeleton, at least, these ossification centers did not appear in all bones at the same time. This made possible an examination of centers in different stages of development in one section of material. It was also apparent that the center of ossification in the tibia did not always have precisely the same appearance in all embryos of the same age. This was also observed in the rat by Bloom and Bloom ('40). However, this is of no consequence here since the enzymatic picture will always be described with relation to the histological picture.

The hyaline cartilage which constituted the skeleton at these early stages was entirely devoid of alkaline phosphatase as indicated by the absence of precipitate after incubation with substrate. The enzyme first made its appearance in the connective tissue surrounding the cartilage in a localized region which was destined to become a center of ossification. Both cellular and intercellular elements exhibited blackening, but the nuclei of the cells had the largest concentration. In areas in which the cells were less closely packed, it was possible to see that the black precipitate was in the form of fine granules. In addition to this, several larger and darker staining granules were evident in many of the nuclei. The cartilage cells at this stage of development did not exhibit significant histological changes and did not contain either enzyme or bone salts (fig. 1).

With the onset of the cartilaginous changes the enzyme became evident in the nuclei and cytoplasm and in the surrounding matrix. The concentration, as indicated by the degree and extent of blackening, was low at first but pro-

gressively increased. The matrix always contained less enzyme than either cytoplasm or nuclei. It was seen to occur as a fine black precipitate. With further degeneration, bone salts appeared in the cartilage matrix. They were visualized in both the control and incubated slides by the ammonium sulfide method and had the form of brown-black granules of varying sizes and were arranged singly or in groups (fig. 2). The enzyme at this time was present throughout the area of degeneration as well as in the surrounding connective tissue (fig. 3,4).

After the beginning of invasion and erosion of the calcified cartilage by mesenchymal elements, glycerophosphatase could be demonstrated in the surrounding connective tissue, the remaining cartilage cells and in the calcified spicules. It is interesting to note that the enzyme disappeared from the spicules after a short time.

By 19–20 days of gestation a collar of bone was well formed around the diaphysis. This bone, along with the remaining spicules of calcified cartilage, was readily evident in the undemineralized control slides. Enzymatic activity was strong in the periosteal connective tissue, osteoblasts, primitive marrow elements and endothelium of the invading vascular tissue. The processes of ossification had proceeded towards both ends of the bones but a considerable amount of cartilage remained. In figure 5 is shown a low power view of the tibia of a late 19-day fetus. The outlines of the small celled cartilage at the end of the bone are clearly seen. No phosphatase is present there. The perichondrial blackening, on the other hand, stands out in marked constrast. Phosphatase is obviously present in the area of enlarged cartilage cells. The distribution is the same as it is within the larger epiphyseal growth zone of the early post-natal bones which will be described later in the text.

II. Post-natal development

A. From 1 to 23 days. The processes occurring during early post-natal life appeared to be a continuation of those

already described for the late fetal bones. However, although only a crude subjective estimation could be made, it appeared that the concentration of enzyme as indicated by the degree of blackening was greater in the post-natal tissues.

During this period of growth there was a progressive increase in the size of the primary marrow cavity and increase in the number of marrow elements. The latter were always phosphatase positive. The compact bone of the diaphysis progressively increased in thickness. Osteocytes lying within it but close to the endosteal border were strikingly positive. Older and more centrally located osteocytes were negative. Blood vessels within the matrix were heavily blackened. The periosteum and endosteum as well as the perichondrium located around the still cartilaginous parts contained enzyme in both cells and fibers (fig. 6).

The epiphyseal growth zone showed three clearly defined regions: (a) non-oriented resting cartilage cells; (b) cells arranged in columns parallel to the long axis of the bone; (c) hypertrophic cartilage cells in which changes similar to those described for the fetal ossification centers were constantly occurring (fig. 12). Glycerophosphatase was not evident in either cells or matrix of the resting cells. A faint precipitate appeared in the outermost oval-shaped columnar cells and increased towards the hypertrophic zone which was heavily blackened (fig. 7). Close to the zone of erosion the nature of the cells was so changed that it was difficult to determine whether the enzyme was present in nucleus, cytoplasm or both. In general, however, it appeared to located in both with a slightly heavier concentration in the nucleus. Enzyme was present in the matrix in the form of densely packed, fine granules.

Osteoblasts lying along the calcified spicules showed enzyme in both nuclei and cytoplasm. In many instances it appeared that a form of uncalcified tissue was present, possibly osteoid. This was always free of enzyme. When incorporated into newly formed bone the osteocytes were also positive and the newly formed bone matrix appeared faintly positive.

Osteoclasts were never observed to contain precipitate and were very difficult to distinguish.

At 7 days of age the first evidence of the formation of a secondary ossification center in the epiphysis appeared. The histological changes were slight and consisted of an enlargement of the lacunae and the usual nuclear and cytoplasmic changes. Enzyme was located in the nuclei and cytoplasm and on the borders of the capsules (fig. 8). By 10 days of age, invasion of the epiphysis by vascular mesenchyme and erosion of the central region had occurred. Enzyme was now found in the connective tissue elements, the endosteal lining, the osteoblasts and vascular elements. The ring of hypertrophic cartilage surrounding the area of erosion of calcified cartilage contained enzyme in the nuclei, cytoplasm and matrix. The zone of non-oriented, resting cartilage located between the epiphyseal ossification center and the columnar cartilage cells of the epiphyseal plate was phosphatase free and stood out in marked constrast to the other two regions. At 15 days erosion had progressed peripherally. The calcified cartilage which still remained was phosphatase negative, while the newly laid bone contained a small amout of enzyme. However, this disappeared and the bone matrix became completely negative. Osteocytes enclosed within the newly formed bone were positive. At this stage the innermost layer of cells of the articular cartilage was positive, enzyme occurring in the nuclei, cytoplasm and inner margins of the capsules. The blackening decreased progressively towards the outer layer of cells which was usually phosphatase free. This was not in agreement with the observations of Lorch ('47) who reported that in rat bones only the inner layer contained enzyme whereas in the mouse the nuclei were strongly positive throughout the depth of the articular cartilage. In order to determine whether this difference in results could be due to age factors, the articular cartilage of all the bones used was examined carefully. Phosphatase was never found in the outer layer. The typical appearance of articular cartilage is shown in figure 9.

By 23 days numerous marrow spaces were present and contained positively staining secondary marrow elements. The lining of the spaces was also strongly positive. Cartilage cells were still present along the outer margins of this region and stained heavily.

B. One month. At one month of age and slightly later the bones were still actively growing. The epiphyseal growth zone was wide, open, and contained much enzyme. The distribution was the same as in the younger bones (fig. 12). The compact bone of the shaft increased in width and was phosphatase free. Close to the endosteal side of the shaft, the osteocytes were positive. Their protoplasmic processes were also markedly blackened and stood out in strong constrast to the matrix (fig. 10). The periosteum was considerably narrower than in the younger bones but still stained heavily. Blackening was present in cells and fibers. Endosteum was thin and also stained heavily.

More cancellous bone was present in the epiphysis. It was phosphatase negative while the endosteal lining, osteoblasts and young osteocytes showed blackening. The vascular and marrow elements were positive as usual.

C. Two months. The most significant change which occurred by two months of age was a decrease in width of the epiphyseal plate due to a decrease in the number of cartilage cells (fig. 13). (Silberberg and Silberberg, '40, reported a similar change in other species of mice.) The hypertrophic region as before contained much enzyme, while the columnar cells had a variable amount depending on their position. Resting cells were not as evident as in earlier stages. Enlarged cartilage cells resembling hypertrophic cells were occasionally present at the junction of the epiphyseal plate and the bone of the epiphysis. These stained very black, especially in the nuclei. The capsules were also strongly positive. In the subepiphyseal layer the bony trabeculae were wider than in the earlier stages and were phosphatase free. The other elements showed the same phosphatase distribution as previously described.

D. Five and one-half months. At 5½ months of age there was no longer much evidence of bone formation in the epiphysis. Only the marrow elements, endosteum and osteocytes lying close to the endosteum were phosphatase positive. Osteoblasts were sparse in number but where found still contained enzyme. Matrix was negative.

The greatest changes occurred in the epiphyseal plate (fig. 14). There was a decrease in the number of cells and evidence of the beginning of formation of a wall of bone separating the epiphysis from the diaphysis. Resting cartilage cells were no longer apparent but the columnar arrangement of cells was still evident in areas. Here there was a heavy blackening of the nuclei, cytoplasm and matrix. Hypertrophic cells were also less apparent. In figure 11 is shown a high power view of the cells typical of the epiphyseal plate at 5½ months. Both the extracellular and intracellular phosphatase distribution are evident.

In the subepiphyseal region a number of large trabeculae were still present. The thin endosteum surrounding them was positive as before and the osteocytes contained within were positive only if very close to the borders. There was a great decrease in the number of osteoblasts but where found they still contained enzyme. Both primary and secondary marrow elements increased in number and, as in younger animals, showed phosphatase. The periosteum surrounding the compact bone of the shaft, in addition to being thinner than that of the one-month old bones, showed enzyme only in the inner layer. The outer layer was completely phosphatase free.

E. Ten months. There was no significant difference in the phosphatase picture in 10-month old bones as compared with 5½-month bones. The numbers of cells in the epiphyseal plate had further decreased. Where present, however, they contained enzyme as did the surrounding matrix.

F. Thirteen months. At 13 months of age only small, scattered groups of phosphatase positive cells remained in the epiphyseal plate (fig. 15). The matrix immediately surround-

ing these cells contained the black granular precipitate but other regions of the matrix were negative. Blood vessels and their contents found in the transverse bony shelf were still strongly positive. The articular cartilage had undergone some retrogressive changes and not all of the inner layer of cells contained enzyme as previously. However, scattered groups stained strongly. In the epiphysis and subepiphyseal region the same distribution of enzyme as noted before was found. The diaphyseal structures also remained the same.

DISCUSSION

The results of this histochemical study were in good agreement with the biochemical analyses of Robison and his coworkers (Robison, '36). Phosphatase was always found in areas about to undergo mineral deposition and increased in amount with progress of the mineralization. The exact localization of the enzyme was of interest. Not only the cells, which might be expected to be centers of activity, but the hypertrophic cartilage matrix and the newly formed bony trabeculae as well exhibited phosphatase activity. It is, of course, possible that its presence in the latter areas might have been due to a diffusion of the precipitate after it was formed. However, this is not likely in view of the exact reproducibility of results and the absence of precipitate outside of the sections or in the lacunar spaces where it would be expected if diffusion were involved. Furthermore. it has been experimentally demonstrated by Danielli ('46) that phosphatase in soft tissues, at least, is in its physiologically normal position.

The occurrence of this extracellular phosphatase was also observed by Lorch ('47) in her demineralized material and was interpreted as supporting the theory of Danielli ('46) that phosphatase has significant activity only in the nuclei and in extracellular sites. The idea that the phosphatase of the cartilage matrix is physiologically active is a plausible one. In the epiphyseal growth zone of the young bones studied here, the concentration of extracellular phosphatase was high

as indicated by the amount of precipitate. Later when the growth processes had slowed considerably, there was a decrease not only in the numbers of phosphatase containing cells but in the amount of enzyme in the matrix surrounding these cells. It must also be pointed out, however, that phosphatase was found in the cytoplasm as well as in the nuclear and extracellular positions. Unless the conditions to which the material was subjected during the histochemical procedure were such that a naturally occurring inhibition was overcome or a missing factor was provided, it must be supposed that the cytoplasmic phosphatase was physiologically active as well.

Among the most interesting of the observations were those of the change in localization of phosphatase coincident with histological change. In the 15-day embryos, enzyme was found only in the perichondrium of the central part of the future diaphysis of the tibia. The concentration was not high. The region which would later ossify contained no enzyme at all. Slightly later the first evidence of histological change in the cartilage occurred and was accompanied by the appearance of enzyme activity. With the progress of histological differentiation towards the epiphyses, there was a corresponding increase in distribution and amount of enzyme. The maxima appeared to be reached during the first month of the post-natal life when the growth processes were also at their height. After two months of age evidence of decreasing activity became apparent. This coincided with the histological changes which have been reported by other authors (Silberberg and Silberberg, '41) and which were noted here. Finally by 13 months of age, considerable decrease in activity had occurred in the epiphyseal ossification center, in the epiphyseal plate, and the endosteum and periosteum.

"The importance of phosphatase in calcification, vigorously supported by Robison, still remains to be demonstrated." (McLean, '43; p. 86.) However, there is no doubt that all the data which have so far been collected very strongly

suggested a significant role for alkaline phosphatase. Certainly the observations reported here of close correlation between histochemical and histological changes support and extend the previous histochemical and biochemical data. Significant among the details was the fact that in the older bones where phosphatase activity decreased sharply, the primary and secondary marrow elements and the lining of the vascular channels, which play no primary role in mineralization, showed no change in enzyme activity.

SUMMARY

1. The histochemical localization of alkaline phosphatase was studied in demineralized mouse bones of different ages from 15 days of gestation to 13 months of age.

2. In the fetus enzyme was present only in centers of ossification. It appeared first in the connective tissue surrounding a definitive ossification center, then in the cartilage cells and matrix and finally in the invading mesenchyme, vascular tissue and osteoblasts.

3. The appearance of enzyme always preceded the appearance of bone salts.

4. Ossification was never observed in the absence of phosphatase.

5. In young post-natal bones, phosphatase was found in the inner layer of cells of the articular cartilage and in the inner borders of the surrounding capsules; in the inner layers of columnar cells and in the hypertrophic cells and matrix of the epiphyseal growth zone; in the calcified cartilage spicules; endothelium of vascular tissue; osteoblasts; young osteocytes and their processes; marrow elements; periosteum and endosteum; and Haversian canals.

6. The secondary centers of ossification in the epiphyses had fundamentally the same distribution of enzyme.

7. Decrease of growth activity in the older bones was marked by a decrease in the number of cells in the epiphyseal plate. Associated with this was a decrease in phosphatase activity. By 13 months of age only a few scattered groups

of phosphatase positive cells remained. The periosteum became narrower and lost the phosphatase in the outer layer. Osteocytes enclosed within the compact bone of the shaft became phosphatase free.

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PLATES

PLATE 1

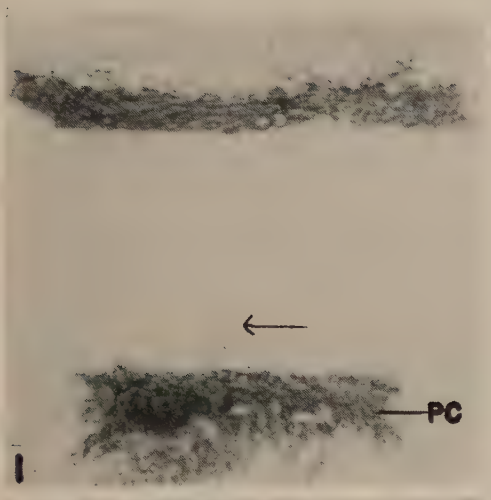
EXPLANATION OF FIGURES

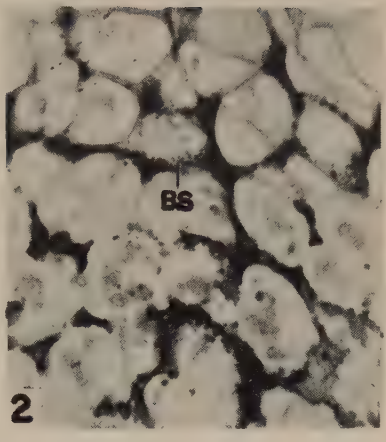
1 Longitudinal section through the primary ossification center of a 16-day fetus. Alkaline phosphatase is not yet present in the cartilage but is quite evident in the perichondrium. Note, however, the enlarged lacunae and slight evidence of blackening in the chondrocytes (marked by arrow) in the lower half of the figure. Incubated one-half hour. PC, perichondrium.

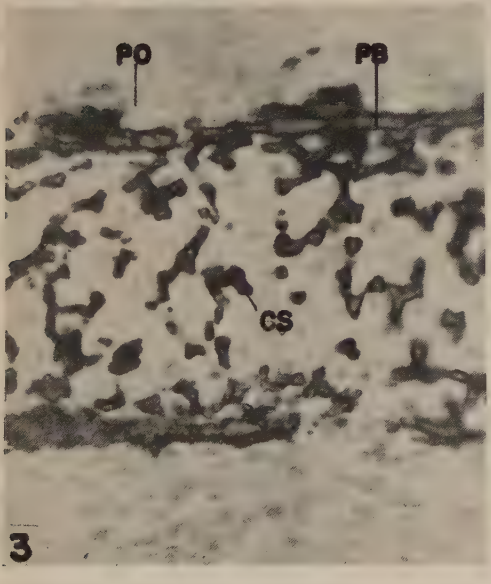
2 High power view of a longitudinal section through the primary ossification center of an 18-day fetus. Unincubated control slide. The blackening is due to the presence of bone salts. Note that in the lower left hand quarter the cartilage matrix has been eroded. BS, bone salts.

3 High power view of a longitudinal section of the primary ossification center of a 17-day fetus. This is an unincubated section which served as control for the incubated section shown in figure 4. The periosteal bone and calcified spicules show well. PO, periosteum; PB, periosteal bone; CS, calcified spicule.

4 Incubated for one-half hour. Compare with control shown in figure 3. Alkaline phosphatase is present in the periosteum, cartilage cells, and very faintly in the cartilage matrix. PO, periosteum; C, chondrocyte.


PC
1


BS
2


PO
PB
CS
3

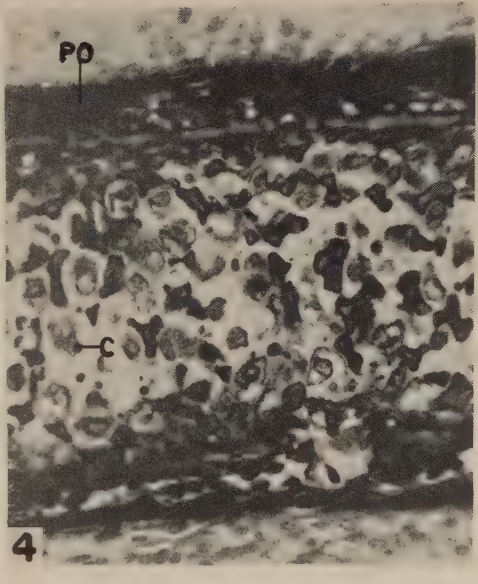

PO
C
4

PLATE 2

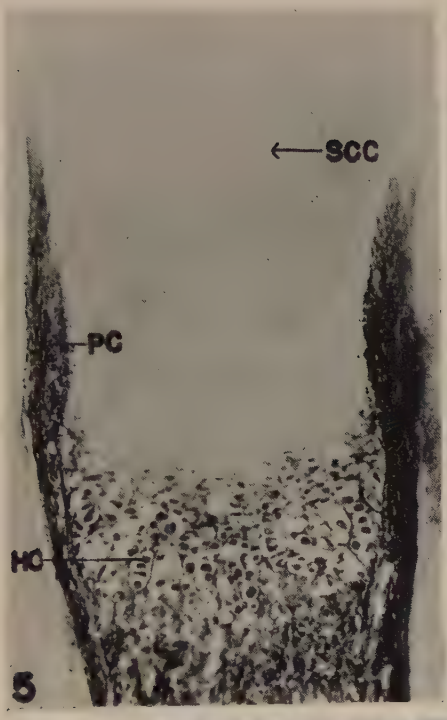
EXPLANATION OF FIGURES

5 Longitudinal section through the distal end of the tibia of a late 19-day fetus. Incubated for one hour. The small celled cartilage is phosphatase free. The perichondrium, periosteum, hypertrophic cartilage cells, and subepiphyseal region have much enzyme. SCC, small celled cartilage; PC, perichondrium; HC, hypertrophic cartilage.

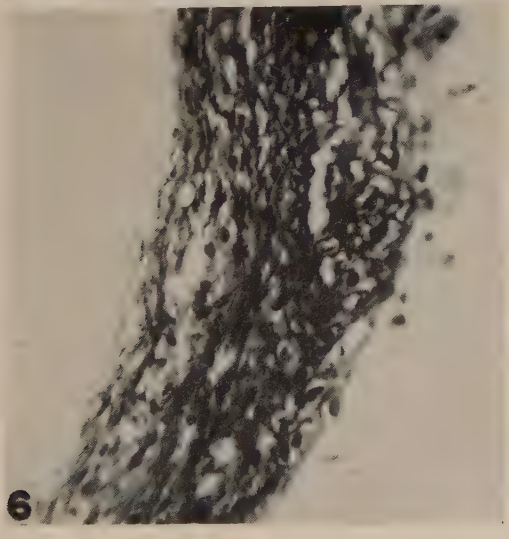
6 High power view of the perichondrium of a one-day old male mouse. The distribution of phosphatase in the cells and fibers is illustrated.

7 Layer of hypertrophic cartilage cells and zone of erosion in proximal end of tibia of three-day old female mouse. Since the section was not completely demineralized, the blackening between the cells is due to the presence of both bone salts and enzyme. Incubated for one-half hour.

8 High power view of the epiphyseal cartilage of a 7-day old male mouse. Incubated for one-half hour. The matrix is still phosphatase free while the cells are strongly blackened.


SCC
PC
HC
5

7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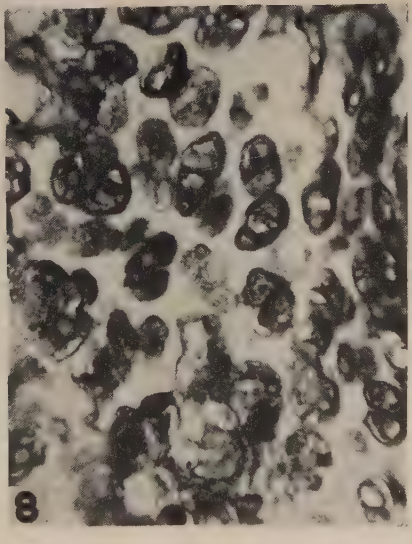
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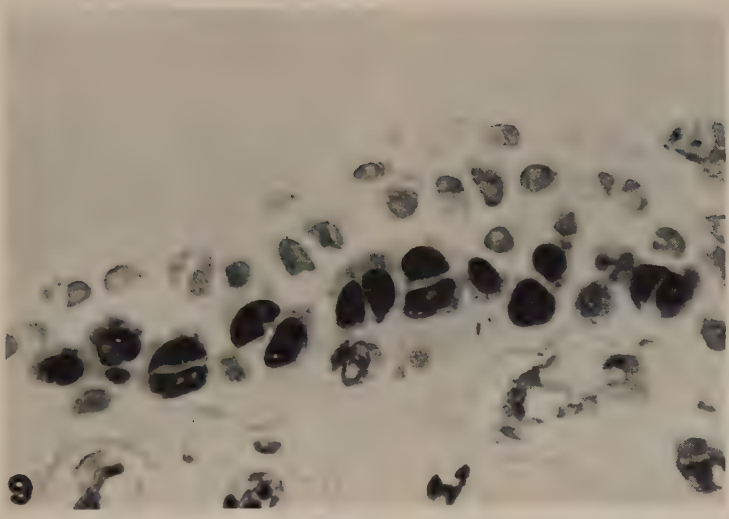
PLATE 3

EXPLANATION OF FIGURES

9 Longitudinal section of articular cartilage of the tibia of a 40-day female mouse. The cells closest to the epiphysis have the largest amount of enzyme as indicated by the degree of blackening. Note also the presence of phosphatase in the borders of the capsules and in the matrix. Incubated for one hour.

10 Longitudinal section of compact bone from 167-day old male mouse. The osteocytes and their processes, and the endosteum have phosphatase activity while the matrix is negative. Incubated for one-half hour. O, osteocyte; OP, osteocyte process; E, endosteum; M, compact bone matrix.

11 High power view of the hypertrophic cell layer of a 167-day old male mouse. Enzyme is present in the cartilage cells and matrix. Incubated for one-half hour.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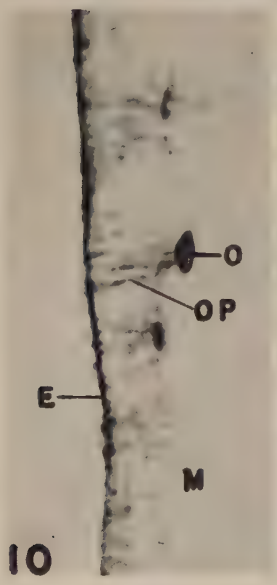
O
OP
E
M
10

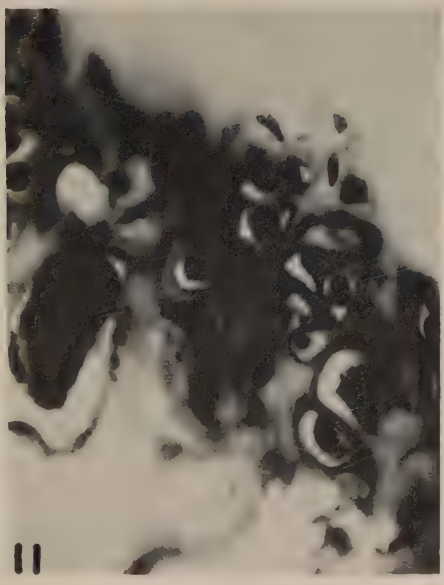
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PLATE 4

EXPLANATION OF FIGURES

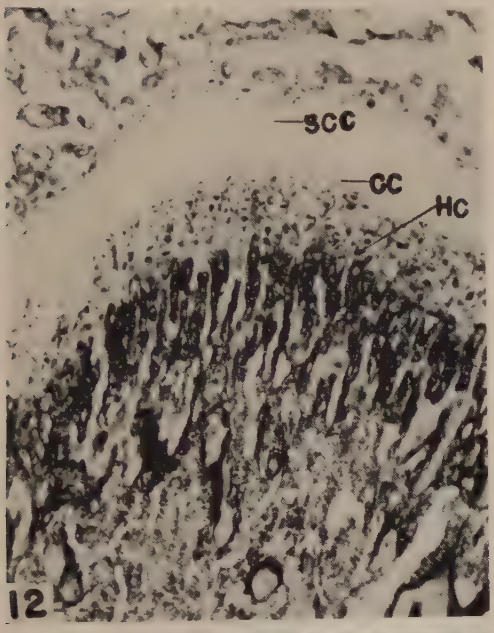
12 Longitudinal section of epiphyseal plate of tibia of 23-day old male mouse. Note the width of the plate. Phosphatase is present in the columnar and hypertrophic cells. The small celled cartilage has negative reaction. SCC, small celled cartilage; CC, columnar cartilage; HC, hypertrophic cartilage. Incubated for two hours.

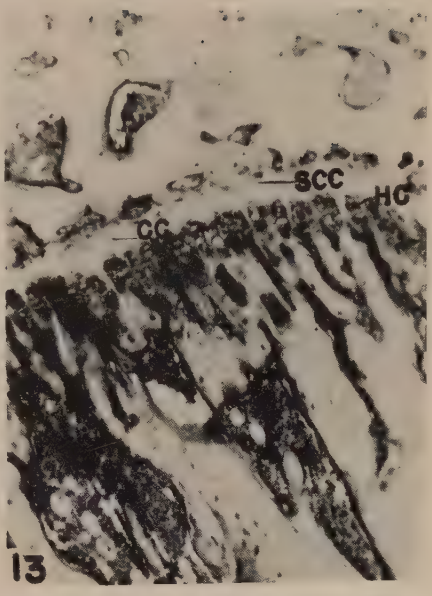
13 Longitudinal section of epiphyseal plate of tibia of 59-day old female mouse. Note decrease in width of plate. Incubated for two hours.

14 Longitudinal section of epiphyseal plate of tibia of 167-day old male mouse. Plate has further decreased in width. There is evidence of the formation of a transverse bony ridge. Small celled cartilage is no longer present. A few phosphatase containing columnar cells can be seen. Hypertrophic cells are strongly blackened. Incubated for two hours. B, bone.

15 Longitudinal section of epiphyseal plate of tibia of 395-day old male mouse. Only scattered groups of phosphatase containing cells remain. Incubated for one and one-half hours. BV, blood vessel.

The magnifications of figures 12–15 are identical.


SCC
CC
HC
12


SCC
HC
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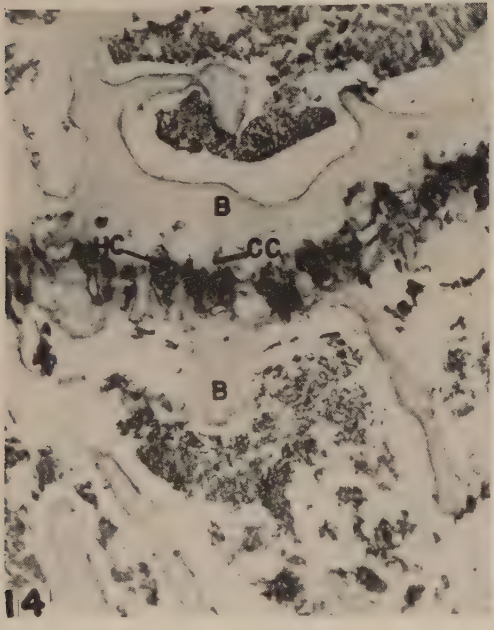
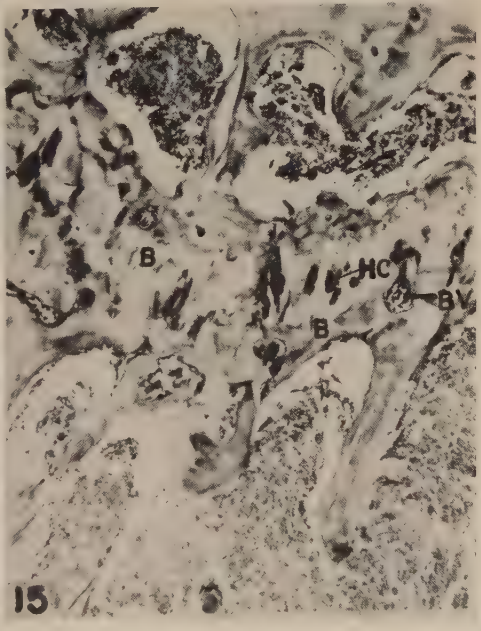

B
HC
CC
B
14


B
HC
BV
B
15

ON THE PRESENCE OF CALCIFIC BODIES, CARTILAGE, BONE, FOLLICULAR CONCRETIONS AND THE SO-CALLED HYALINE BODIES IN THE HUMAN PITUITARY[1]

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TWENTY-TWO FIGURES

INTRODUCTION

Large highly calcified concretions were observed by Plaut and Galenson ('44) in 5 of the 110 human pituitaries examined. All 5 were from adults between 37 and 60 years old.

Costero and Berdet ('39) say that the most frequent foreign elements in the human pituitary are the mesenchymal derivatives such as connective tissues which may become fibrous, irregularly hyaline with cartilagenous nodules, with or without normal architecture, or even osseous trabeculae that are grouped into imperfectly organized lamellae.

Concretions in the follicles of the human pituitary have been described especially by Erdheim ('04) and by Plaut and Galenson ('44). The latter authors say that colloid con-

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The writer also wishes to thank Mr. Tamir Nassar, Technician, for assisting in the preparation of the material and Mr. Haigaz Artinian, Photographer, for making the photomicrographs.

cretions are a constant finding in the anterior pituitary lobe and in the pars intermedia of the human fetus and newborn, but that most of them disappear during the first postnatal months.

Plaut and Galenson clearly differentiated between concretions found in the follicles and those associated with the capsule. Shanklin (46a) described the development, histology and senescence of the capsular concretions. Their sex ratio and incidence were discussed by Rasmussen ('33) and by Shanklin ('48). The latter writer proposed the term follicular concretions for those in the follicles and the term mesothelial concretions for those of the capsule, since the latter are derived from the arachnoidal mesothelium.

Herring ('08) found small highly refractile hyaline bodies in the posterior lobe of a number of adult animals. Herring thought that they represented secretion which passed up the stalk to enter the third ventricle. Many later writers referred to these hyaline masses as the bodies of Herring. Tello ('12, figs. 10 and 11) found hyaline bodies in the neurohypophysis which he interpreted as degenerating nerve endings and refers to these bodies as balls of degeneration. Bucy ('32) reviewed the evidence regarding these hyaline bodies. He rejects the interpretation of Herring and agrees with Tello that they are degenerated end bulbs of the nerve fibers.

It is the purpose of this paper to describe the occurrence and histologic appearance of calcified bodies, cartilage, bone, follicular concretions and the so-called hyaline bodies observed in 100 human pituitaries.

MATERIALS AND METHODS

The composition of this material has been previously described (Shanklin, '46a, '46b, '47 and 48). A table including sex, age, cause of death and a summary of the histological findings was published (Shanklin, '48) hence will not be repeated in this paper.

Observations on calcific bodies

In addition to the fairly numerous mesothelial concretions, with lamellae having moderate amounts of calcium, other bodies with a high degree of calcification were found in 7 of the pituitaries in our series. These were found in the following pituitaries: M118, a 14-year female; M123, a 70-year male; M176, also a 70-year male; M210, a 60-year male; M223, a 49-year male; M238, a 4-year female; and M244, a 36-year female.

Examples of calcific bodies, with well defined lamellae are included in figures 1 to 3. The one in the first figure is one of a number of similar bodies located in an area of inflammatory cells that have destroyed a large part of the infundibular process. Other areas (figs. 2 and 3) are situated in the capsule of the neurohypophysis near the insertion of the stalk. The one in the first figure has only the inner lamellae highly calcified, whereas in the other two most of the lamellae are filled with calcium while the interlamellar material had disappeared. That is especially true of those in figure 2.

Although highly calcified masses were usually observed in older pituitaries they also occasionally occur in younger specimens, for example, specimen M238 is from a 4-year old female and M118 from a 14-year old girl.

Besides calcified concentric bodies in the capsule other calcified masses were found in the neurohypophysis. A single peculiar shaped calcified body with a stem was seen in a 36-year old female (M244). As shown in the photomicrograph (fig. 4) the center of the stem has a lighter stained filament that continues into the center of the body where it is coiled. The body itself has indistinct striations. In another specimen, a 70-year old male, (M123), three small irregularly shaped poorly laminated calcified bodies were seen in a cleft deep in the infundibular process.

Two small laminated calcified bodies were seen in the hypothalamus of a 40-year old male (M91). These were near the midline adjacent to the eminentia medialis.

Observations on cartilage and bone

A single piece of hyaline cartilage was observed in the infundibular process in a specimen (M238) from a 4-year old female. This was the only pituitary in our series in which the cartilage was found. The cartilage (fig. 5) lies in an irregularly shaped cleft and is completely cut off from the capsule. On one side the chondrocytes are much smaller and resemble those seen in passing from cartilage to the perichondrium. Two isolated large chondrocytes are located in the tissue on the side of the cleft away from the large piece of cartilage.

Bone was observed in 10 pituitaries (M109, male, 43 years; M115, male, 40 years; M182, female, 6 years; M192, male, three years; M206, male, 40 years; M210, male, 60 years; M223, male, 49 years; M228, male, 48 years; M242, male, 45 years and M244, female, 36 years).

Bone in the pituitary is not always asssociated with old age for it was found twice in children; one a three-year old male, the other a 6-year old female.

A single piece of bone was observed in each of two specimens, several pieces were seen in 4 pituitaries and numerous pieces in the remaining 4.

Although bone was found associated with the neurohypophysis in 10 specimens it was only observed once in the anterior lobe. This specimen (M109) had one piece of bone in the anterior lobe and several pieces in the stalk.

Bone was found in the stalk of three specimens (M109, M115, M242). The remaining 7 had bone associated with the infundibular process. One of these, with numerous irregularly shaped pieces of bone deep in the infundibular process, is shown in figure 6. A long narrow piece of bone located deep in the infundibular process is included in figure 7. This is one of a number of pieces of bone in this pituitary. An area of inflammatory cells lies along one side of this piece of bone (fig. 7, i.c.)

TABLE 1

Table giving specimen number, sex and age of pituitaries containing follicular concretions with their location, degree of lamination and variety or varieties of lining cells

NO. OF SPECIMEN	AGE AND SEX	A = ANT. I = INT.	DEGREE OF LAMINATION			LINING CELLS		
			Scant	Medium	Marked	Acido-phils	Baso-phils	Chromo-phobes
M86	F, 45	A	+					
M91	M, 40	A	+					
M92	M, 65	A		+			+	
M95	F, 59	I			+			+
M98	F, 80	A		+				+
M100	M, 65	A		+			+	
M101	M, 37	A			+		+	+
M106	M, 42	I			+		+	
M107	M, 4	A		+		+		+
M115	M, 40	I		+			+	
M117	F, 52	A + I		+			+	+
M119	F, 31	A	+			+		+
M123	M, 70	A	+					+
M164	M, 52	A			+		+	+
M170	M, 65	A + I			+		+	+
M171	F, 18	A			+			
M172	F, 83	A		+			+	+
M176	M, 70	A	+					+
M178	F, 72	A			+		+	+
M204	M, 40	A + I			+			+
M206	M, 35	A		+				+
M207	M, 48	A		+				+
M213	F, 41	A	+			+		+
M214	M, 16	A	+					+
M215	F, 12	A	+			+	+	
M217	M, 40	A			+		+	
M220	M, 43	A	+			+	+	
M223	M, 49	A + I		+		+	+	+
M224	F, 52	A	+				+	+
M226	M, 45	A		+			+	
M227	F, 21	I		+				+
M228	M, 48	A		+			+	
M230	M, 23	I			+			+
M232	M, 40	I		+				+
M233	M, 86	A		+				+
M235	M, 45	A		+			+	+
M244	F, 36	A		+			+	
M246	M, 20	A			+			+
			10	17	11	6	19	25

One long narrow piece of bone, located in a fold of collagenous fibers that penetrated deep into the neurohypophysis, had a thin zone of newly formed bone at its periphery. A single incomplete layer of osteoblasts surrounded the newly formed bone.

One wedge shaped piece of bone was found lying outside the capsule, with the pointed end penetrating through the entire capsule into the underlying parenchymatous tissue. Neither here, nor in any of the other specimens did the tissues react to the bone as to a foreign body.

Observations on follicular concretions

Incidence, sex ratio and age distribution (table 1). Our observations on follicular concretions do not include pituitaries from the newborn and the first three years of life as these were described by Plaut and Galenson ('44). Of the total 100 pituitaries in our series 71 of them were from individuals between the ages of 4 and 86 years. One or more follicular concretions were found in 38 (53.5%) of the 71 pituitaries. Of the 71 pituitaries 25 were from females and 46 from males. Follicular concretions were observed in 13 (52.0%) of the 25 pituitaries from females and in 25 (54.3%) of the 46 from males.

An analysis is next presented to determine whether or not follicular concretions occur more frequently in old age. As the above analysis failed to show a significant sex difference specimens from both sexes are grouped together. The 71 pituitaries were divided into three age groups, 4 to 19, 20 to 39 and 40 to 86 years. There were 18 pituitaries from the youngest group, 16 in the middle group and 37 in the oldest group. Of the 18 pituitaries in the youngest group 4 (22.2%) had follicular concretions, the 16 in the middle group had 7 (43.7%) and the 37 in the older group had 27 (73.0%).

Histology (table 1, figs. 8 to 13). Follicular concretions were found exclusively in the pars anterior in 28 specimens, only in the pars intermedia in 6 and in both parts in 4 pituitaries.

Only those masses with some degree of lamination are included as follicular concretions, however, the degree of stratification varies considerably. Of the 38 pituitaries with follicular concretions, the concretions in 10 had poorly developed laminae, 17 medium and 11 well developed layers. No cells were seen in the center of the concretion nor were differentiated membranes observed between the lamellae. Neither central nuclei nor specialized masses were observed in the center of the concretion. Sometimes the central part of the concretion is stained more darkly than the peripheral portion (fig. 9), however in some the central is more lightly stained part (fig. 8). The lamellae did not differ markedly in their calcium content. In general the material in the concretions was finely granular and lightly stained with the eosin. The material in all of the layers appeared to be essentially the same. A few of the follicular concretions had scattered granules of brown pigment. These were usually located between the lamellae.

The cells lining the follicles containing the concretions are those normally found in the epithelial parts of the pituitary. The lining cells about one of the most typical follicular concretions in each of the 38 positive pituitaries is recorded in table 1. The follicular lining cells observed were as follows: chromophobes only, 13 times; basophils only, 8 times; acidophils only, none; chromophobes and basophils, 8 times; chromophobes and acidophils, three times; basophils and acidophils, two times, and all three varieties once. Typical laminated concretions were observed (M86, M91 and M171) three times without any lining cells. The lining cells of several of these follicular concretions were thickened in places and thin in others (figs. 10 and 12).

Observations on concretions in Erdheim glands

Four concretions were observed in the glands of Erdheim ('04) in specimen M248 from a 3.5-year old girl. These were moderately calcfied bodies with well formed lamellae (fig.

22). Two of the concretions were in the interstitial tissue of the gland while the other two were in the lumina of the acini. In one the glandular epithelium was normal except on one side where the concretion caused flattening of the epithelium. All the epithelium of the second acinus was flattened by the concretion in its lumen.

Observations on hyaline and granular bodies

Age distribution and incidence. Hyaline bodies, with a modified center, were observed in 17 pituitaries in our series. Although most of them were recorded it is very probable that some were overlooked. They usually occur in the older pituitaries, for 12 of the 17 were from individuals 40 years or older, however, three were from children aged 1.6, 3.5 and 13 years old.

Location in pituitary. All of them were observed in the neurohypophysis. The two favored sites being proximal to the pars intermedia and at the insertion of the stalk into the infundibular process. However, some were seen in the distal part of the infundibular process. Hyaline bodies appeared to be especially numerous in those specimens with extensive basophil invasion of the neurohypophysis and they tended to be near the area invaded.

Varieties of bodies in neurohypophysis. At least three varieties of non-cellular bodies are found in the neurohypophysis. These are: masses of colloid material of irregular shapes and sizes, hyaline bodies with a central core surrounded by a zone of granular material and lastly, round bodies containing fairly evenly dispersed granules surrounded by a thick membrane. Only the latter two varieties will be described here.

The typical hyaline body (figs. 14 to 19) has a large round central core of fairly homogenous material which is always more darkly stained than the peripheral layer. Usually the core stains a shade of purple after hematoxylin and eosin and the line of demarkation between the two areas is

always sharp. Although the core is always approximately round the body itself may be round to oval.

Modifications of the central core and of the peripheral zone were observed. In some bodies the central part of the core was more lightly stained than the outer part. The outer darker part of the core varied in thickness. It ranged from a broader dark zone to a thin rim of darker material, however, this darker band appears to be of the same composition as the central part. In some it resembles a nuclear membrane (figs. 14 to 17). Occasionally a sharply defined, thin, homogeneous refractile membrane surrounded the central mass.

The shape of the hyaline masses varied considerably. Some were nearly round, others oval, while some were of irregular shape. A few had a tendency to be lobulated at the periphery and a number of them showed some degree of scalloping of the outer border (fig. 17). The central core was at times eccentrically placed (figs. 15 and 17). There was considerable variation in the amount of material in the outer layer for in a few it was very scant (figs. 14 and 18) while in others it was abundant (figs. 15 to 17). The peripheral layer consists of fine evenly distributed very closely packed granules. These granules stain purplish pink. At times a thin well defined refractile membrane was observed around the periphery of the mass. This membrane, even when present, did not always completely surround the body. A few normal nuclei were at times observed closely applied to the exterior of the body, however, there appears to be no significant relationship between the two. There is no reaction whatever of the cells and fibers in the vicinity of these bodies.

What appear to be younger stages were frequently observed. The smallest of these were small round or oval bodies, having granules identical with those in the larger bodies, but no modified core (figs. 18 and 19). Others had a slightly darkened center but no limiting membrane. Some were very small but had a darker central mass surrounded by a thin darker zone (fig. 19). All of these appear to consist of essentially the same material as the larger ones.

The bodies included in figures 14 to 17 were 26 × 42, 36 × 43, 42 × 54 and 26 × 40 μ, respectively. The smaller one included in figure 20 was 24 × 33 μ.

All of the bodies included in figures 14 to 20 may not be identical, especially that in figure 20. The latter is smaller and has a more darkly stained center than most of the others.

In contrast to most of the larger hyaline bodies other round to oval masses of granules, surrounded by a thick membrane, are frequently observed. These bodies (fig. 21) have fairly evenly dispersed granules with no suggestion of a modified central area. In pituitaries with abundant pigment these bodies may contain coarse pigment granules, however, the granules are usually small and are not more eosinophilic than the cytoplasm of the surrounding cells in the neurohypophysis. These granules should be classified as amphophilic as they are not stained by a specific dye. The membrane is usually thick and fairly eosinophilic. A pyknotic nucleus is occasionally seen associated with the membrane.

DISCUSSION

The pituitary concretions of earlier investigators should be divided into two major varieties: mesothelial and follicular. The two differ from one another both in their origin, development and in their histology. The writer (Shanklin, '46a, figs. 1 to 7) described the development of mesothelial concretions from the arachnoidal mesothelium. This mesothelium is found most frequently associated with the capsule in the region where the stalk is inserted, however, more recent studies (Shanklin, '48, figs. 2 to 8) show that both mesothelium and mesothelial concretions may be found inside the pituitary. Mesothelial concretions begin their formation around one or more hypertrophied mesothelial cells. The normal cytoplasm of these cells is then replaced by a hyaline material and the surrounding mesothelial cells form processes which encircle the enlarged cell. Following this stage alternate layers of hyaline material, separated by membranes, are laid down. The outermost layer in some of the older meso-

thelial concretions is surrounded by a thick capsule of collagenous fibers (Shanklin, '46a, figs. 8 to 12).

The follicular concretions are formed in the lumina of the follicles of the anterior lobe. They appear to consist of finely granular material that may or may not be stratified. Plaut and Galenson ('44) seldom found distinct lamination of the follicular material in embryos up to 29 cm crown-heel length, whereas in the older fetal specimens and the newborn about half of the concretions were more or less laminated. In contrast to the mesothelial concretions none of the follicular concretions were formed around a central cell and none of them had formed membranes separating the lamellae.

The highly calcified laminated bodies observed in the human pituitary are usually associated with the capsule near the insertion of the stalk (figs. 2 and 3). Their location, size, shape and the arrangement of their lamellae clearly show that most of these calcific bodies are the remains of degenerated mesothelial concretions. The lamellae of the different mesothelial concretions vary greatly in the degree of their calcification. This is illustrated by examining the figures previously published (Rasmussen, '33, fig. 4, Shanklin, '46a, figs. 8 to 10, and '48, figs. 1 to 9). The writer previously described the older mesothelial concretions as undergoing a "melting down" during senescence ('46a, figs. 11 and 12). In the light of our subsequent studies we conclude that a few of the mesothelial concretions, become so highly calcified that when the hyaline material degenerates the highly calcified layers remain.

Most likely some calcified bodies in the pituitary do not have mesothelial concretions as their precursors. For example, the one included in figure 4 and the three described in a cleft in the neurohypophysis (M123). The calcific masses described by Plaut and Galenson ('44) appear to be associated with follicular rather than with mesothelial concretions.

None of the calcified bodies observed in our material were phleboliths. Rasmussen ('33, fig. 3) described a concretion

within a blood vessel in the infundibular process of a normal male.

Our next problem is to explain the presence of extraneous mesodermal derivatives in the neurohypophysis.

No mesodermal components were seen in the neurohypophysis of the 60-mm pig after staining with Hortega silver carbonate (Shanklin, '44, figs. 1 to 3) however, blood vessels and the accompanying mesoderm is present by the 125-mm stage (fig. 4). In the 180-mm pig there is extensive mesodermal invasion of the neurohypophysis (figs. 7 and 8). No mesodermal derivatives were seen in the neurohypophysis of the 3-month human fetus (Shanklin, '40, fig. 3), however, connective tissue trabeculae were observed projecting into the neurohypophysis of the 5½-month human fetus (figs. 4 and 7).

We agree with Costero and Berdet ('39) that mesodermal derivatives are among the more numerous extraneous elements in the pituitary. Although Costero and Berdet describe cartilage and bone in the human pituitary they do not give its incidence. In our series of 100 pituitary hyaline cartilage was observed in a single specimen while bone was seen in 10 pituitaries. Bone was found chiefly in the stalk and the infundibular process and only once in the anterior lobe. The precursors of bone are found in all parts of the pituitary, but bone was usually limited to the neurohypophysis. Most of the pieces of bone observed were located completely within the neurohypophysis.

Neither the calcific bodies nor the hyaline cartilage and bone were associated with the proliferation of connective tissue such as is seen in advanced fibrosis of the human pituitary. Inflammatory cells were observed in two of the pituitaries having bone and in those cases bone formation may have been associated with the pathology of the area.

Whatever the stimulus may be that causes ossification in the human neurohypophysis this stimulus is not limited to old age for bone was observed in two children.

The follicular concretions were found exclusively in the epithelial portions of the pituitary and all except three were surrounded by follicular cells. Although Plaut and Galenson say that these concretions disappear in the first fetal months our study shows that they frequently reappear. Our material suggests that the follicular concretions in the older pituiaries, although much larger, are esentially the same as in the fetus and newborn.

We failed to find any significant sex difference in the occurrence of follicular concretions. There was, however, a definite increase in their incidence with advancing age. According to Plaut and Galenson these concretions are formed in the fetus under the influence of maternal hormones.

Concretions, which appear to correspond to the human follicular concretions, have been described in the pituitary of the elephant (Stendell, '14, fig. 84; Wislocki, '40, and Hanstrom, '46a, fig. 13). Stendell included in his figure 84 comparative stages in the development of these concretions. Hanstrom ('47, fig. 15) also found laminated bodies in a large cyst within the pars intermedia of the badger.

Plaut and Galenson ('44) found a single concretion in a gland of Erdheim. This concretion was partly intracellular (fig. 17). All four of the concretions observed by us were in the glands of Erdheim in a 3.5-year old girl. Two were located in the lumina of the glands. Plaut and Galenson assume that these concretions are formed from the secretion of the gland. Our observations support that assumption.

Herring ('08) says that the appearance of the small highly refractile hyaline bodies in the posterior lobe (figs. 14 and 15) suggests that of cells which have undergone hyaline degeneration. According to him these hyaline bodies have a distinctly granular appearance, and are not unlike the granules of the cells of the anterior lobe, but do not stain as deeply. He thought they represented secretion that passed up the stalk to enter the third ventricle. Although many writers agreed with his conclusions and referred to these as the bodies

of Herring, many other investigators disagree with that interpretation.

Tello ('12, figs. 10 and 11) found hyaline bodies, similar to those of Herring, in the neurohypophysis which he interpreted as degenerating nerve endings. He refers to these as balls of degeneration. According to him these bodies frequently have a reticulated center and often part of the axon is still found attached.

Bucy ('32) agrees with Tello that the hyaline bodies of the pars nervosa are degenerated end bulbs of the nerve fibers. He includes one of these in figure 6A stained by Cajal's silver pyridine method. Van Dyke ('36) includes a lengthy discussion on the nature of these bodies and also rejects the interpretation of Herring. Hanström ('46a, fig. 10) demonstrated bulb- or pear-shaped nerve endings in the pars posterior of the tiger after the Bodian method. Hanström ('47, fig. 8) shows a bulb-like nerve ending within the pars posterior of the polar bear in an azan preparation. He states that these bodies may attain a size up to 40 μ in the tiger and up to 35 μ in Ursus maritimus. He also says that Romeis ('40, p. 441) gives their diameter in man as up to 15 μ. The bodies described in our human material are considerably larger than those measured by Romeis.

Hanström thinks that these bulb-like nerve endings may be connected with the hormone production of the pars posterior. He says ('47, p. 16, fig. 8) that the bulb is usually surrounded by a pit containing a coagulum which stains red-grey with azan while the bulb is stained blue. He found within the bulb itself small intensely red-stained droplets. Droplets of the same size and color were observed in the pit around the bulb. Two of our figures (figs. 18 and 19) show smaller bodies near the larger mass. According to the interpretation of Hanström these may represent secretory material from the end-bulb itself.

The fascinating idea of neurosecretion was discussed at length by Scharrer and Scharrer ('45) and more recently by Collin ('47), hence will not be dealt with further in this paper.

Romeis ('40) described in the human neurohypophysis masses which he called x- and y-bodies. Hanström ('46b, fig. 9) found in the posterior lobe of the shrew (Sorex araneus) a y-body. One body included in our figure 20 differs from the other bodies and may correspond to the x- or y-bodies of Romeis. Hanström ('47) found numerous scattered bodies in the neurohypophysis of the polar bear that did not correspond either to the "Herring bodies" or the "x- and y-bodies" of Romeis.

Although well defined bodies are found in the neurohypophysis of many animals following a number of fixatives and many staining methods, Gersh and Tarr ('35) using fixation by freezing and drying failed to demonstrate segregated masses or hyaline bodies in the neurohypophysis and concluded that such bodies are artefacts.

A comparison of the so-called hyaline bodies included in figures 14 to 19 in this paper, from the human, with those included in the figures by Hanström, from various mammals shows that the two are similar. Although the two are similar our studies neither confirm nor deny the nerve-ending interpretation. The infrequency of these bodies in the older human pituitaries and their rarity in younger pituitaries, following staining by hematoxylin and eosin casts some doubt on the nerve-ending interpretation. The conclusion of Gersh and Tarr that these bodies are artefacts also complicates the problem of their interpretation.

The smaller encapsulated bodies are entirely different from the larger so-called hyaline bodies with a central core. Those filled with pigment granules were only observed in the neurohypophysis of specimens having abundant pigments. Their granules were obviously derived from the pigment bearing cells. The more lightly stained amphophilic granules are similar in appearance to the granules in some of the hypertrophied pituicytes, the glands of Erdheim, the older basophils and the tumorettes. The similarity of the granules in the above structures was discussed by the writer (Shanklin, '47) in a study on the origin of tumorettes in the human

neurohypophysis. These bodies appear to be remnants of cells which have lost their nuclei and cytoplasm leaving behind a thickened cell membrane filled with granules.

Conclusion. In conclusion we can state that most of the highly calcific bodies in the human pituitary are derived from degenerated mesothelial concretions. Mesodermal derivatives such as cartilage and bone were observed in the neurohypophysis. Follicular concretions, lined by one or more varieties of the parenchymatous cells, were found in the anterior lobe. These concretions are numerous in the newborn, are sharply decreased in number during infancy and childhood and become more abundant in the older age groups. The so-called hyaline bodies were found in the human neurohypophysis. They were similar in their morphology to those previously published from various mammals. We conclude that the granules in the small encapsulated bodies are derived from pigmented and other cells in the neurohypophysis. These bodies appear to be degenerating cells which leave their granules, after the loss of their cytoplasm, surrounded by a thickened membrane.

SUMMARY

1. Complete serial sections of 100 human pituitaries varying from the newborn to 86 years of age, were systematically examined for calcific bodies, cartilage, bone, and the so-called hyaline bodies.

2. Calcific bodies were observed in 7 pituitaries. Their morphology shows that most of them are the remnants of highly calcific mesothelial concretions that have undergone senescence.

3. Hyaline cartilage was observed once and bone was found in 10 pituitaries.

4. Follicular concretions were found in 38 of the 71 pituitaries from persons between 4 and 86 years. These concretions are numerous in the newborn, decrease during early childhood and increase again in old age.

5. The follicular concretions are surrounded by one or more varieties of the anterior lobe parenchymatous cells.

6. Concretions were observed in the glands of Erdheim in a single pituitary.

7. The so-called hyaline bodies are described and their interpretation as nerve endings is discussed.

8. Small bodies in the neurohypophysis, filled with granules identical with those in the pigment and other cells in that structure, surrounded by a thick membrane, are described.

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PLATE 1

EXPLANATION OF FIGURES

All are photomicrographs of sections from the human pituitary and are stained with hematoxylin and eosin.

1 Calcified concretion in the infundibular process of a 14-year old female. × 425.

2 Calcified bodies in the pituitary capsule of a 70-year old male. × 425.

3 Calcified bodies in the pituitary capsule of a 60-year old male. × 425.

4 A peculiarly shaped calcified body in the infundibular process of a 36-year old female. × 425.

5 An area of hyaline cartilage in the infundibular process of a 4-year old female. × 130.

6 Pieces of irregular shaped bone in the infundibular process of a 6-year old female. × 130.

7 A long narrow piece of bone in the infundibular process of a 49-year old male. i.c., area of inflammatory cells. × 130.

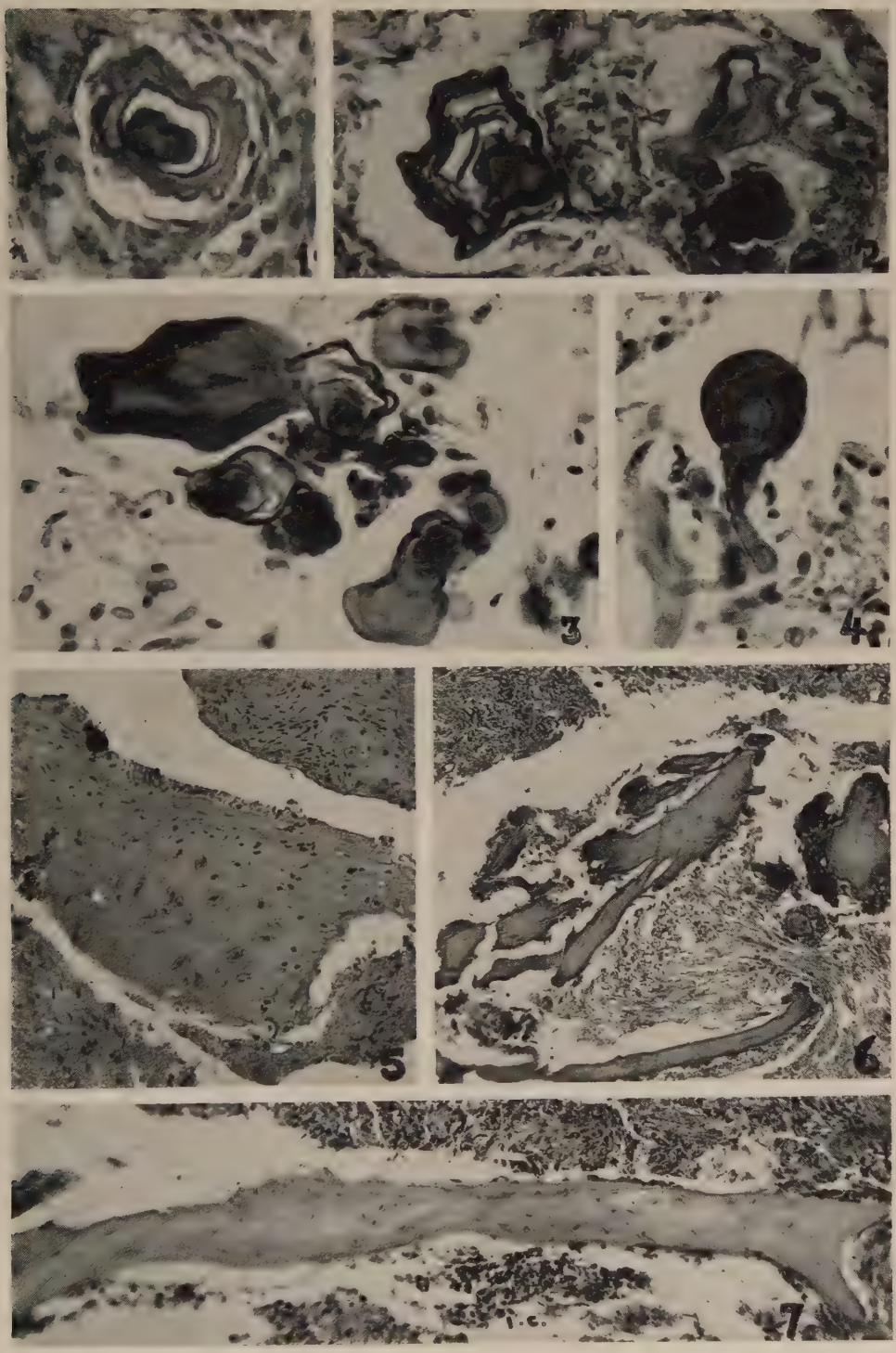
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PLATE 2

EXPLANATION OF FIGURES

8 Follicular concretion lined by chromophobes and a few acidophils in the center of the pars anterior of a 40-year old male. × 425.

9 Follicular concretion lined by basophils in the distal part of the pars anterior of a 40-year old male. × 425.

10 Follicular concretion lined by basophils in the proximal part of the pars anterior of a 45-year old male. × 425.

11 Follicular concretion lined by chromophobes and eosinophils in the distal part of the pars anterior of a 72-year old female. × 425.

12 Follicular concretion lined by chromophobes and esoinophils in the distal part of the pars anterior of a 65-year old male. ×425.

13 Colloid filled follicles under the capsule in the distal part of the pars anterior of a 20-year old male. × 425.

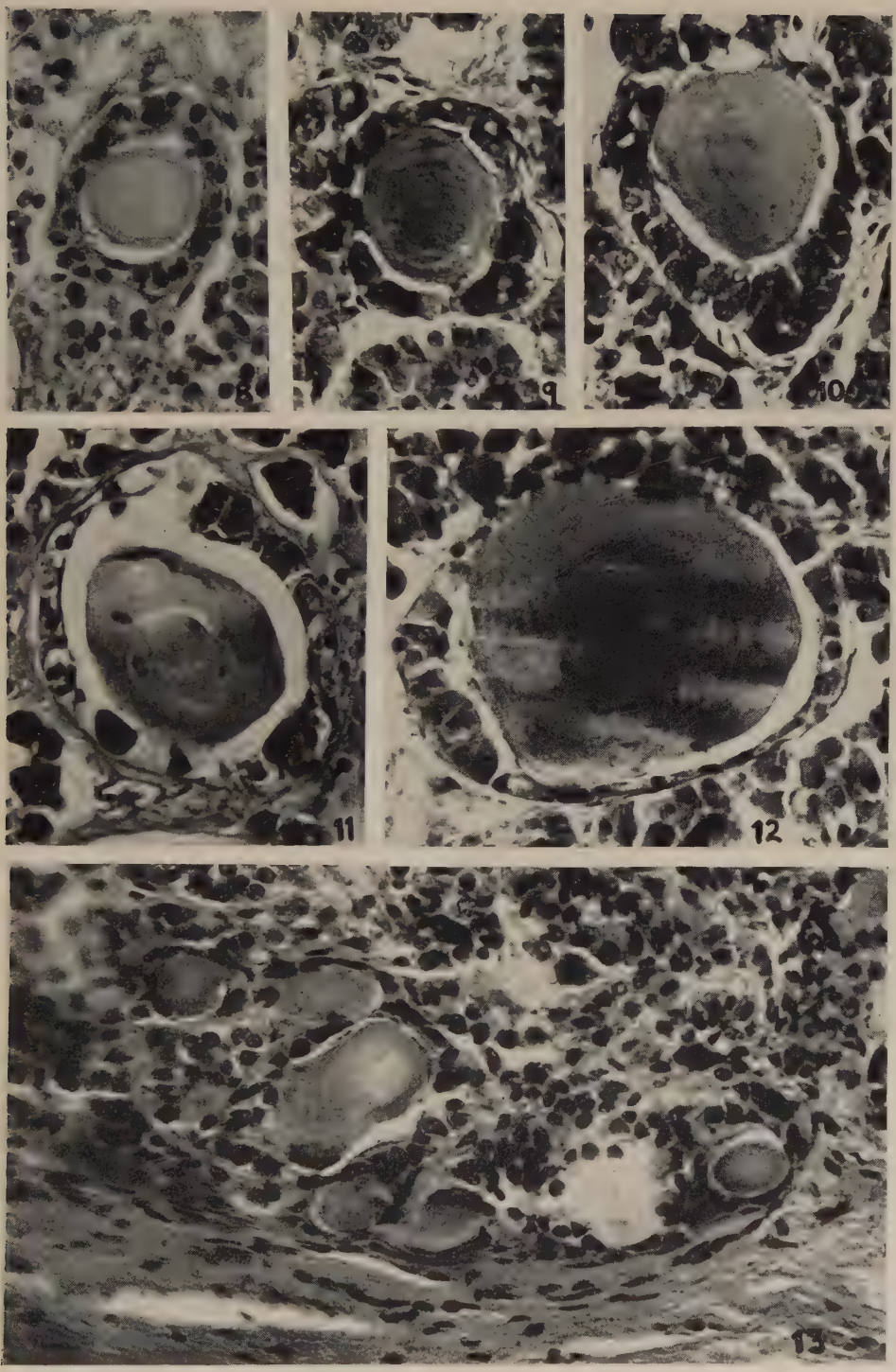
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PLATE 3

EXPLANATION OF FIGURES

14 A hyaline body in the proximal part of the infundibular process of a 3.5-year old female. × 425.

15 A hyaline body at the junction of the stalk and the infundibular process of a 1.6-year old female. × 425.

16 A hyaline body at the junction of the stalk and the infundibular process of a 40-year old male. × 425.

17 A hyaline body in the center of the infundibular process of a 60-year old male. × 425.

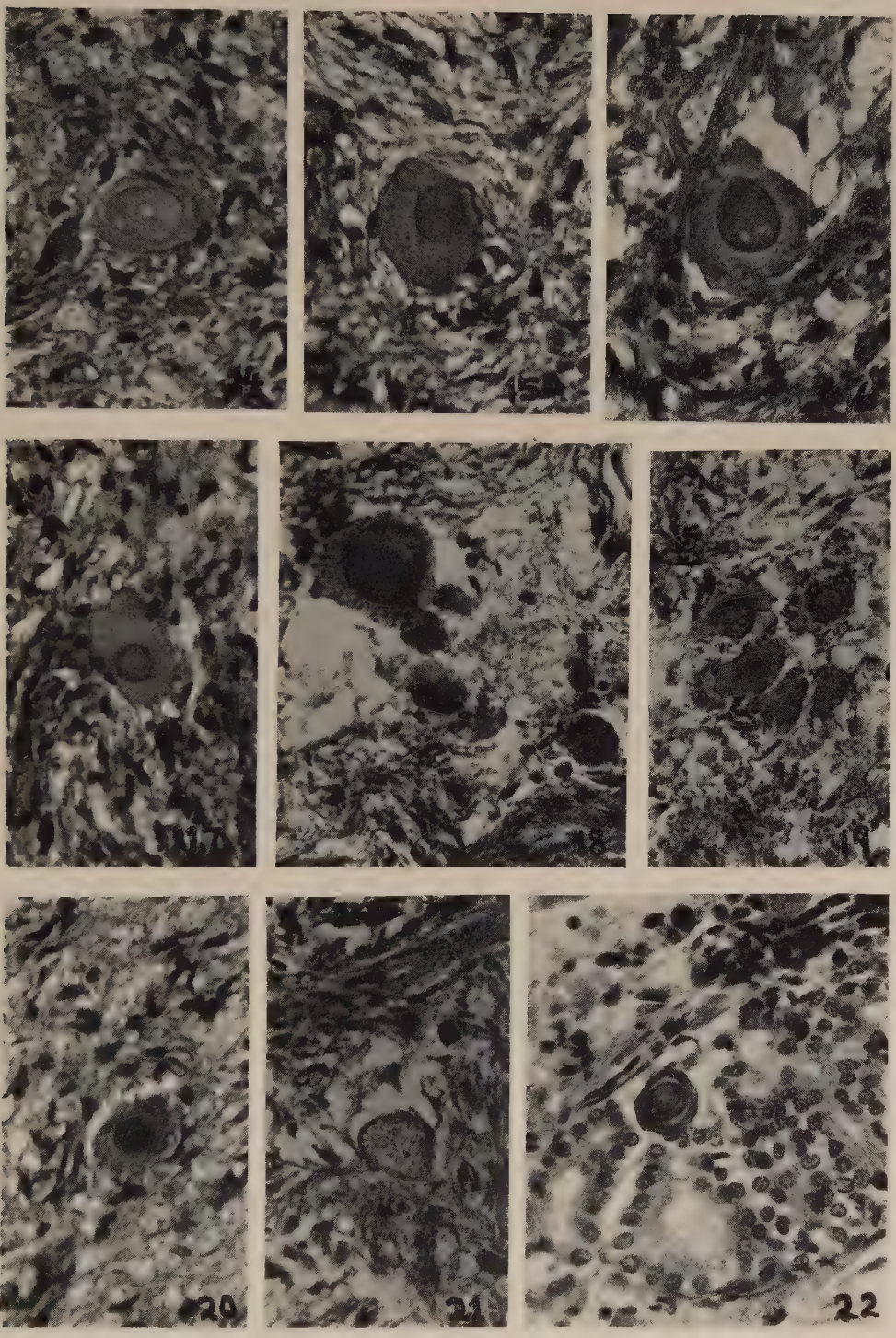
18 A group of hyaline bodies at the junction of the stalk and the infundibular process of a 51-year old male. × 425.

19 Another group of hyaline bodies at the junction of the stalk and the infundibular process from the same pituitary as that in the preceding figure. × 425.

20 A hyaline body in the proximal part of the infundibular process of a 40-year old male. × 425.

21 An encapsulated body containing amphophilic granules in the infundibular process of a 65-year old male. × 425.

22 Concretion in a gland of Erdheim in the infundibular process, near the par intermedia, of the same pituitary included in figure 14. × 425.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SOME EFFECTS OF NITROGEN MUSTARD ON THE DEVELOPMENT OF EXTERNAL BODY FORM IN THE FETAL RAT [1]

DAVID HASKIN

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FOURTEEN FIGURES

INTRODUCTION

The sulfur and nitrogen mustards have proven useful in the study of certain developmental processes. Their characteristic effects simulate in many respects those produced by x-rays and other penetrating radiation. Three aspects of their activity are pertinent to the experiment reported here.

1. Mitotic activity is inhibited by action upon the interphase nucleus. Friedenwald and coworkers ('47) applied nitrogen mustard in graded quantities to corneal epithelium and made histological studies of the effects. The tissues exposed to higher concentrations were found to be depleted of mitotic figures for several days although cells undergoing mitosis at the time of exposure completed the process and were subsequently arrested in the resting stage.

2. The most actively proliferating cells may be specifically inhibited. Gillette and Bodenstein ('46) and Bodenstein ('47) established that, at appropriate concentrations of nitrogen mustard, those cells undergoing most rapid proliferation at the time of treatment are especially susceptible to mitotic

[1] This research has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. The problem has been conducted under the guidance of Dr. Carl R. Moore. Acknowledgments are made to Dr. John Hutchens for a supply of the chemical employed for treatment.

arrest. Amphibian larvae of various ages were treated and their subsequent development was analyzed both grossly and cytologically. Younger individuals appeared most severely affected. In the central nervous system of older forms, "the non-dividing, differentiating cells are not affected by the agent and continue unhindered their processes of differentiation, while the cells capable of dividing enlarge greatly under the influence of the agent but cease to multiply."

3. The action of nitrogen mustard is a rapidly completed one. Karnofsky and his coworkers ('48) occluded the blood vessels leading to the hind limbs of rats and rabbits. Immediately following application of the clamps nitrogen mustard was injected intravenously into more anterior vessels. The clamps were removed after periods of two to 15 minutes following injection and the animals autopsied a few days later. Bone marrow which is highly susceptible to the agent was found to be aplastic in the exposed anterior region and essentially unaffected in the "protected" hind limbs.

The above mentioned specific effects of the nitrogen mustards upon rapidly proliferating tissues suggested their use as agents for selective inhibition of mammalian fetal structures. With the use of fixed dosages and exposure of fetuses to the agent at particular stages in ontogeny, the structures undergoing most rapid development at the time of treatment should be differentially inhibited. The following experiments were undertaken to expose fetal rats to nitrogen mustard via the maternal circulation.

MATERIALS AND METHODS

Previously bred female albino rats were used for the preliminary experiments on non-pregnant animals in order to establish suitable concentrations of the agent for treatment. Virgin female albino rats from a strain inbred at the University of Chicago for over 20 years were mated with males of the Sprague-Dawley strain at witnessed copulations. Pairs mated in the evening were caged together overnight and the age of embryos was calculated from midnight.

Of the nitrogen mustards, methyl-bis (beta-chloroethyl) amine has been most thoroughly investigated and was utilized in this study in the form of its soluble hydrochloride salt. Gilman and Philips ('46) and others have reviewed the history, chemistry, and pharmacology of the compound, and reviews of its clinical and pathological actions have recently been published (see Rhoads, '46; Jacobson et al., '46; Karnofsky et al., '48; Graef et al., '48). To insure uniform potency of this unstable chemical, fresh aqueous solutions of HN_2 were prepared for each series of injections and treatment was completed within 10 minutes of preparation. The nape of the neck was chosen as the site of subcutaneous injections after intestinal adhesions had been noted in animals treated intraperitoneally. Precautions were taken to avoid skin contact with HN_2 because of its considerable vesicant action.

Single injections were administered either on day 12, 13, 14 or 15 of pregnancy and all fetuses were removed at sacrifice on day 21. Weight and crown-rump length were determined after preservation in Bouin's fixative.

Fourteen of the 20 gravid females survived to autopsy on day 21 and 9 of these carried live fetuses; 6 such litters contained fetuses exhibiting abnormalities.

A few abnormal fetuses were cleared by the Schultze-Dawson technique for the study of skeletal deformities.

EXPERIMENTS

I

A series of preliminary experiments with non-pregnant animals was undertaken to determine the maximum single dose of HN_2 that could be administered to an animal with survival in satisfactory condition. One milligram per kilogram of body weight was selected on the basis of data presented in table 1. This quantity is slightly less than 40% of the LD_{50} as determined by Graef et al. ('48).

The clinical symptoms observed were in essential agreement with the findings of other investigators for the dosage range

employed. Of note was the occurrence of extensive edema about the site of injection, severe mucoid diarrhea, weakness, loss of appetite and weight from approximately the second to the 8th day following treatment. The high incidence of mortality at the three- to 6-day period is associated with pathological changes of the intestinal epithelium, profound

TABLE 1

Single doses of nitrogen mustard in distilled water injected subcutaneously at the nape of the neck

NO.	WEIGHT IN GM	MG HN_2 PER KG BODY WT.	APPROX. SURVIVAL AFTER INJECTION
1	215	4.6	2 days
2	186	3.1	3–4 days
3	164	3.0	3–4 days
4	180	2.8	3–4 days
5	220	2.7	3–4 days
6	193	2.6	3–4 days
7	204	2.5	3–4 days
8	172	2.4	3–4 days
9	190	2.2	3–4 days
10	186	2.2	3–4 days (s.w.m.)
11	196	2.0	3–4 days (s.w.m.)
12	196	2.0	3–4 days (s.w.m.)
13	216	1.8	3–4 days
14	172	1.2	5 days (s.w.m.)
15	164	1.2	19 days (s.w.h.)
16	172	1.2	6 days (s.w.m.)
17	188	1.1	12 days (s.w.h.)
18	202	1.0	12 days
19	190	1.0	19 days (s.w.h.)

(s.w.m.) sacrificed when moribund.
(s.w.h.) sacrificed when apparently healthy.

lymphatic atrophy and general depression of the hematopoetic system. The dead bodies of 4 rats were partially cannibalized by their less affected cage mates; a very rare occurrence in this colony.

II

Pregnant rats were treated with HN_2 in unit doses of 1 mg per kilogram body weight, or half of that dose, on either day

12, 13, 14, or 15 of gestation. Fourteen rats survived until day 21 at which time pregnancy was terminated by sacrifice, the uteri examined and the young preserved. Results of this experiment are summarized in table 2.

All fetuses in rats treated on day 12 were either dead and badly macerated or entirely resorbed. Three of the 4

TABLE 2

Nitrogen mustard in distilled water injected subcutaneously into pregnant rats on days 12, 13, 14, and 15 of gestation

LITTER NO.	WEIGHT IN GM	DOSAGE MG/KG	DAY INJECTED	NO. OF IMPLANTATIONS	AV. WT. OF FETUSES	AV. CROWN-RUMP LENGTH	NO. ABNORMAL OR RESORBED
1	228	1.0	12	10	All fetuses resorbed		
2	190	1.0	12	7	All fetuses dead and necrosed		
3	200	0.5	12	6	All fetuses dead or resorbed		
4	248	0.5	12	9	All fetuses dead or resorbed		
5	256	1.0	13	..	Adult died on day 20		
6	230	1.0	13	11	0.95 gm	1.70 cm	6 abnormal 5 resorbed
7	254	1.0	13	13	1.39 gm	2.49 cm	11 abnormal 2 resorbed
8	260	1.0	13	11	All fetuses resorbed		
9	242	0.5	13	9	2.39 gm	3.18 cm	6 abnormal
10	230	1.0	14	7	2.19 gm	2.85 cm	3 abnormal
11	242	1.0	14	11	2.65 gm	3.18 cm	none
12	252	1.0	14	11	1.92 gm	2.72 cm	3 abnormal 8 slightly abn.
13	212	0.5	14	12	2.27 gm	3.08 cm	5 abnormal
14	230	0.5, 0.5	14, 15	13	2.23 gm	3.00 cm	none
15	240	0.5, 0.5	15, 16	12	3.01 gm	3.32 cm	none

rats which received injections on day 13 carried litters having deformed live young, and one litter was completely resorbed. The fetuses exposed to half of the unit dose were less severely affected.

Of the 4 rats injected on day 14, three carried litters containing some abnormal fetuses while the 4th litter contained

only grossly normal fetuses. No resorptions were noted in the group of females treated on day 14. Two rats were given half doses on two successive days without visible effect upon their young.

Although the number of animals utilized in this experiment is too small for statistical analysis, a sharp time gradient of sensitivity to HN_2 is suggested by the failure to obtain live fetuses from females injected on the 12th day of gestation, the lower fetal mortality of those treated on day 14, and the greater size of fetuses in the latter group.

A wide range in the severity of abnormality was observed among individuals in each affected litter. Alterations in the external body form of at least two fetuses from each litter will be described; one, exhibiting the greatest deformity and the other showing the least.

DESCRIPTIONS OF FETUSES

Litter no. 6

Dosage: 1 mg HN_2 per kilogram body weight injected on day 13

Fetus no. 4 (most deformed in this litter) may be seen in figures 5 and 12; it weighs 1.13 gm and has a crown-rump length of 2.18 cm. The head is proportionally small and appears deeply set between the shoulders. It bears a 3.0 × 3.5 mm cranial fault in the occipital region, markedly underdeveloped jaws and a short, barrel-shaped body. The anterior limbs are bilaterally adactylous with two minute knobs on each stump representing the missing distal portion of the limbs. Of the hind limbs, the left is curved toward the anal region and the right is straight and lies over the genital area. Each hind limb has three rudimentary toes. The tail is considerably shorter than normal.

Fetus no. 2 (least deformed in this litter) is shown in figure 6. Weight 0.90 gm, crown-rump length 2.00 cm. This fetus is somewhat less affected than its littermates in that the cranial fault is less pronounced, the posterior limbs have

normal posture, and the tail is of normal length. Except for a missing right toe, the hind feet appear only slightly malformed. The head and anterior limbs and feet, however, exhibit approximately the same degree of defect as in its more abnormal siblings.

All members of litter no. 6 exhibit considerable malformation. Figure 2 may be compared with figure 1 (an apparently unaffected fetus from a mother exposed to only a half-dose) to illustrate the typical marked reduction in size of the more severely affected fetuses. Each one has a cranial fault in the occipital region, receding lower jaw, virtual or complete adactyly of the anterior feet and a variety of postural and digital deformities of the posterior limbs. In this litter as in all others exposed to HN_2 on day 13, the development of foot structures appears to be more severely modified in the anterior limbs than in the posterior limbs.

Litter no. 7

Dosage: 1 mg HN_2 per kilogram body weight injected on day 13

Fetus no. 3, weight 1.34 gm, crown-rump length 2.34 cm (see fig. 14). Except for a remarkable bilateral posterior curvature of the hind limbs, and a longer tail the malformations produced on this fetus are very similar to those in the most deformed member of litter no. 6.

Fetus no. 9, weight 1.52 gm, crown-rump length 2.50 cm. This fetus differs from its above littermate in that the cranium does not have an occipital fault and the hind limbs have normal posture.

Virtually all fetuses in this group carry the cranial defect, underveloped jaws, short body and severe limb anomalies. As in the previously described litter treated on day 13, failure of normal toe development is more pronounced in the anterior than in the posterior feet. Figure 8 (fetus no. 5) shows clearly the maximum defect in anterior limbs while figure 10 (fetus no. 4) illustrates a lesser degree of modifi-

cation of the hind limb and toes. An example of hind limb torsion in a fetus of this litter is shown in figure 13.

Litter no. 9

Dosage: 0.5 mg HN_2 per kilogram body weight injected on day 13

Fetus no. 2, weight 2.72 gm, crown-rump length 3.32 cm. The head, body, tail and posterior limbs are not deformed. Each hind foot bears two short, but recognizable toes.

Three fetuses in this litter are grossly normal in external form; abnormalities in their littermates are limited to absence and deformity of digits on the anterior feet. This litter was exposed to half the dose of those previously described and the effects are proportionally diminished.

Litter no. 12

Dosage: 1.0 mg per kilogram body weight injected on day 14

Three fetuses in this litter exhibited deformities of similar degree and nature. The remaining 8 fetuses were, with the exception of 1.0 × 2.0 mm cranial faults, essentially normal. One of the former will be described.

Fetus no. 11 may be seen in figures 4 and 7. Its weight is 1.29 gm and crown-rump length 2.29 cm. The head is small, deeply set between the shoulders and displays a 5.0 × 5.0 mm fault over the midbrain. The jaws are underdeveloped and the body short and stout. Each anterior limb is terminated by two proportionally large toes (see fig. 7) and both hind limbs are adactylous, lacking in the usual flexures and folded over the anal region. The tail is short.

A sibling bore the largest cranial fault obtained (fig. 3) measuring 5.0 × 6.5 mm. Fetus no. 1 of this litter shown in figure 9 clearly illustrates the differential effects on limb development obtained from treatment on day 14. The anterior toes are all in evidence whereas the hind toes are repre-

sented by a single structure. Figure 11 reveals the deformities in posterior aspect.

Litters nos. 10 and 13 having been treated on the 14th day with unit and half-unit doses respectively are remarkable only in that about half of each litter exhibited various degrees of syndactyly on the hind feet and in some cases absence of a toe.

In addition to the alterations in external body form described above, examination of the oral cavity of selected fetuses subjected to HN_2 in unit doses on day 13 or day 14 revealed cleft palate of the post-alveolar-process type.

It should be noted that in the majority of fetuses exposed to HN_2 on day 14 of gestation digital deformities are restricted to the hind limbs, and that in no instance is the degree of deformity in the anterior limbs equal to or greater than in the posterior limbs. This is exactly in reverse of the findings in fetuses exposed on day 13. The number of fetal deaths and incidence of anomaly formation is also lower in the group injected at the later time.

DISCUSSION

The results of this study, presented briefly on the preceding pages, demonstrate that a single injection of a water soluble nitrogen mustard compound, methyl-bis (beta-chloroethyl) amine hydrochloride, into a pregnant rat on the 13th or 14th day following copulation induces marked gross abnormalities in the fetuses. Examination of the uterine contents at autopsy one day prior to parturition (20 days + 12 hours post-copulation), and thus 6 to 7 days following treatment, reveals a number of placentae with brown, necrotic borders. The fetuses are of considerably subnormal size and display such gross abnormalities as receding lower jaws, cranial faults, cleft palate, deformed limbs, absence and fusion of digits, shortened tails and unusual body proportions.

Deformities similar to these have been obtained in several species by a variety of techniques. Certain of these anomaly-

producing mechanisms will be commented upon in the light of their possible influence on the above experiment.

A discussion of the literature concerning anomalous development traceable to hereditary factors is beyond the scope of this paper. A few classical observations will be noted to illustrate their diversity. Wright ('22) developed a highly inbred strain of guinea pigs which expressed progressively greater inhibition (to the point of complete suppression) of head and limbs. Hamburger ('35) crossed two species of European salamanders (*Triton taeniatus,* female and *Triton cristatus,* male) with two of the resulting offspring exhibiting bilateral hypodactyly of the hind limbs. The inheritance of chondrodystrophy and its characteristic limb deformities in the "Creeper" fowl have been extensively investigated (Dunn, '39; Landauer, '32; Hamburger, '42). Chesley ('35) has studied a short-tailed mutant of the house mouse. Bagg ('29) and Bagg and Little ('24) subjected mice to x-radiation prior to mating and subsequent generations of these mice bore eye, ear, and vascular defects, deformed heads, short snouts and clubbed feet. Syndactyly, hypodactyly, club foot, abnormal cranium and palate formation are known hereditary defects in man (Mohr, '39, '41; Müller, '37, and Tredgold, '37). However, the rôle of heredity in the study reported here is not of obvious consequence.

Innumerable experiments have demonstrated that deviation of normal morphogenesis may be induced by alteration of the fetal environment. Of the many extrinsic factors known to disturb the course of development, maternal vitamin deficiency (especially riboflavin) and high energy irradiation of the fetuses produce defects in the young of rats which most closely resemble those produced by exposure to nitrogen mustard.

Warkany, Nelson, and Schraffenberger ('41, '42, '43, '44) in a brilliant series of experiments induced congenital malformations in the young of female rats which were reared and bred on a diet deficient in riboflavin. Among the deformities obtained were such skeletal changes as shortness of the

tail, mandible, radius, ulna, tibia, and fibula; fusion of the ribs, syndactylism of the fingers and toes, and cleft palate. Indeed, some of the fetuses startlingly resemble those described in this paper. Addition of the missing dietary fraction as late as the 13th day of gestation prevented appearance of the malformations in the offspring. With histological evidence that the defects occur before or near the onset of chondrification and the knowledge that chondrification in the rat begins on the 14th and 15th days of fetal life they conclude the following: "The malformations are determined not before the thirteenth day and not after the fifteenth day of gestation. A critical stage exists then in the 13 or 14 day rat embryo in which the presence or absence of sufficient riboflavin is of a decisive influence on the development of the embryo."

Are the effects of HN_2 upon fetal development produced essentially indirectly by malnutrition in the pregnant female? A period of about 6 days of general debilitation and appetite loss following injection of HN_2 was observed though an adequate diet [2] was always available to the treated rats. However, failure of other investigators (Nelson et al., '47) to reproduce Warkany's results utilizing a diet probably containing slightly more riboflavin would indicate that a very severe depletion is necessary. Warkany's own breeding experiments with rats receiving alternately deficient and supplemented diets suggest that adult riboflavin reserves would not be depleted with sufficient rapidity to influence membranous bone formation or early chondrification in HN_2 treated rats. (See Warkany and Nelson, '42.) The possibilities that HN_2 inactivates riboflavin directly or disrupts its utilization at the cellular level have not been investigated. Another factor must be considered in relation to fetal nutrition: that of placental damage. Subjectively, a greater area of brown, necrotic tissue was observed around the periphery of the placentae of the more grossly affected fetuses.

[2] This colony is maintained on a diet of "Purina Laboratory Checkers" supplemented with green vegetables.

Whether or not the increased placental necrosis was primary or parallel to fetal defectiveness could not be determined. Transient malnutrition is therefore not easily eliminated as a factor in the above experiments.

The deleterious effects of high energy radiations upon fetuses *in utero* were noted by several biologists and clinicians in the early 1900's. Bailey and Bagg ('23) reviewed the literature in this field prior to 1923. Hanson ('23) x-rayed rats in late pregnancy and produced eye defects, changes in skull shape, and "considerable retardation in growth in some cases." Bagg and his collaborators irradiated fetal rats by exposing the pregnant female to either gamma or roentgen rays and by injection of radioactive salt solutions. Traumatic and developmental defects in the soft tissues were observed. Murphy and de Renyi ('30) exposed the abdominal regions of pregnant rats to graded doses of x-rays. At dosages in excess of 400 r there was a progressive increase in per cent of club feet and absent toes in the young. Job, Leibold, and Fitzmaurice ('35) irradiated the unprotected abdominal regions of gravid rats at known stages of gestation and observed defective brains, eyes and jaws among the surviving offspring. A critical range of dosage was established below which no defect was produced and above which death ensued. A "critical period" of maximum susceptibility to x-ray induced malformation was found for each affected structure: 9th day for the brain, 10th day for the eye, and 11th day for the jaw. Individual variation of degree and type of defect among members of a given litter was attributed to the presence of fetuses in slightly different stages of development at the time of treatment (after the findings of Huber, '15). Kaven ('38) has conducted a similar set of experiments upon mice.

Warkany and Schraffenberger ('47) repeated the experiments of Job et al., utilizing a larger number of rats, a greater range in fetal age and more thorough investigative techniques. Use of large single doses of x-rays resulted in anomaly formation as late as the 16th day of gestation. Deformities in fetuses exposed at a given age were more

severe as the dose increased. Overlapping, but definable "critical periods" of sensitivity of individual structures to x-ray induced malformations were also noted, though they did not correspond to those observed by Job et al. Gross inspection of the defective fetuses revealed: parietal cranial faults with encephaloceles, shortness of the jaws, cleft palates, syndactyly, hypodactyly, club feet and short tails. As in the case of the fetuses malformed by maternal malnutrition these closely resemble the young of HN_2 treated rats. However, cleared specimens revealed certain differences in the pattern of skeletal deformity that distinguish the malnutrition from the x-ray treated fetuses. The few cleared young of rats exposed to HN_2 show alterations in skeletal development that are not characteristic of either group. A detailed report on our findings must await further analysis.

This striking similarity in effects from treatment with nitrogen mustards and high energy radiations is but another in a series of diverse effects common to both agents. The generalization of Bergonie and Tribondeau that the sensitivity of cells to radiation varies directly with their reproductive capacity and inversely with their degree of differentiation obtains as well for the mustards. For this reason, application of certain mustard derivatives to tumor chemotherapy has met with some success (Jacobson et al., '46). Both x-rays and the mustards are highly effective agents for induction of mutations, the latter producing an abundance of chromosomal aberrations. Enlargement of the nuclei in ectodermal cells observed in amphibian larvae exposed to HN_2 has also been noted (Ewing, '26) among the sequelae to large doses of radium as, "a strictly specific feature of cell damage from irradiation which has never been satisfactorily explained." Bodenstein and Kondritzer ('48) analyzed the nucleic acid content of HN_2 treated amphibian larvae and found that, as in x-rayed tissue, synthesis of desoxyribonucleic acid had been blocked while ribonucleic acid production continued. However, too great emphasis upon the similar biological effects of these very dissimilar agents is unwarranted. The differ-

ences in their activity are merely less dramatic. The fact that HN_2 demonstrates a destructive effect on tumors that have become insensitive to treatment by radiation suggests action upon separate phases of cell metabolism. More subtle, but equally cogent differences are described by Friedenwald and coworkers ('47).

The gross resemblance between fetuses bearing anomalies resulting from abnormal genetic make-up, exposure to ionizing radiations, and nitrogen mustard treatment may be resolved in terms of a general inhibiting action of all three agents. Landauer's study ('32 and others) of a genetically determined limb anomaly in fowl led him to conclude, "The skeletal peculiarities of the Creeper fowl (and probably of chondrodystrophy in general) are not brought about by specific factors acting upon the growth of certain parts of the skeleton, but by agencies which influence body growth in general. These agencies must realize their influence at a time when the growth rate of the extremities is at a maximum, thereby exerting a specific inhibitory action upon the formation of these parts of the body."

A like set of conclusions was proposed by Stockard ('21) following his classical observations on the etiology of malformations in the minnow *Fundulus* after exposure to several inhibitory agents.

The observation was made that fetuses from rats injected on day 13 exhibited greater deformity of the anterior limbs than the posterior limbs and that injection on the 14th day reversed this condition; the anterior limbs and digits of fetuses exposed on day 14 may be almost normal in structure whereas the hind limbs and feet may exhibit gross defects. The following factors are suggested as operative in producing this effect:

1. In the sequence of events leading to the formation of a complete limb, a certain portion lies within a "critical period" of sensitivity to inhibition. This sequence may be similar to the proximo-distal wing part determination in the chick (Saunders, '47).

2. The "critical period" may be characterized by a sharp, but transient increase in the rate of cell division. Such an increase may take place during the formation of the foot plate and toe anlagen at the distal end of the forelimb on the 13th day of fetal life.

3. Posterior limb development in the rat lags behind that of the anterior limb by approximately one day (Long and Burlingame, '38). Thus the apparent shift in maximum sensitivity to HN_2 from the fore to the hind limbs may be accounted for.

SUMMARY

Modifications in the external body form of fetal rats produced by exposure to methyl-bis (beta-chloroethyl) amine hydrochloride have been described.

Following preliminary experiments, pregnant albino rats were injected with 0.5 mg/kg and 1.0 mg/kg doses of HN_2 on the 12th, 13th, 14th, and 15th days of gestation. The surviving fetuses were removed at autopsy on day 21, fixed, and examined. Abnormalities observed include:

1. Subnormal size and weight.
2. Cranial faults in the occipital region.
3. Underdevelopment of the jaws.
4. Cleft palate.
5. Absence or various degrees of fusion of toes on the anterior and posterior limbs, with hemimelia as the extreme condition.
6. Abnormal posture of the hind limbs.
7. Shortness of the tail.

These deformities are interpreted as symptoms of a general retardation of development at a critical period in fetal life.

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PLATE 1

EXPLANATION OF FIGURES

Photographs of rat fetuses removed at autopsy 20 days + 12 hours post-copulation from mothers given single injections of the nitrogen mustard, methyl-bis (beta-chloroethyl) amine hydrochloride, on day 13 or 14. See text for descriptions.

1 Fetus of normal size and conformation from litter no. 13 which received a half-unit dose (0.5 mg/kg body weight) on day 14.

2 Fetus no. 6, litter no. 6; the smallest obtained. Mother treated on day 13. This fetus and the remainder of those illustrated were exposed to unit doses of HN_2 (1.0 mg/kg body weight).

3 Fetus no. 1, litter no. 12; treated on day 14. This fetus bears the largest cranial fault observed.

4 Fetus no. 11, litter no. 12; treated on day 14.

5 Fetus no. 4, litter no. 6; treated on day 13.

6 Fetus no. 2, litter no. 6; illustrating differential inhibition of the forelimb.

7 Fetus no. 11, litter no. 12; treated on day 14.

8 Fetus no. 5, litter no. 7; treated on day 13 and exhibiting the extreme forelimb defect typical of the group.

9 Fetus no. 1, litter no. 12; treated on day 14 and illustrating differential inhibition of the hind limb.

10 Fetus no. 4, litter no 7; treated on day 13.

11 Fetus no. 1, litter no. 12; treated on day 14.

12 Fetus no. 4, litter no. 6; treated on day 13.

13 Fetus no. 1, litter no. 7; treated on day 13.

14 Fetus no. 3, litter no. 7; treated on day 13 and bearing the most extreme degree of supination of the hind limbs obtained.

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EFFECTS OF ESTRADIOL AND PROGESTERONE ON LACTATION [1]

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SIX FIGURES

Estradiol, testosterone, androstenediol, androstenedione, dehydro-iso-androsterone and pregnant mare's serum (PMS) inhibit lactation in normal but not in ovariectomized rats (Barsantini and Masson, '47). Since these hormones are strongly luteotropic, it has been suggested that some relationship exists between the pregnancy type corpora lutea formed under the influence of the treatment and the inhibition of lactation (Masson and Barsantini, '48). Many observations (reviewed by Barsantini and Masson, '47) have clearly demonstrated that the active corpus luteum prevents the onset of lactation. Since corpora lutea are normally present during lactation, it may be assumed that the latter are physiologically different from those produced by the steroids or by PMS or those present during pregnancy.

Although progesterone is secreted by large corpora lutea as indicated by the formation of deciduomata (Fluhmann and Laqueur, '42) it cannot be considered responsible for inhibition of lactation since large doses do not influence lactation in normal rats (Folley and Kon, '38; Barsantini and Masson, '47). Recent histologic studies of the uterus from lactating

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rats treated with PMS revealed that, according to the criterion of Atkinson and Hooker ('45) there is a high level of progesterone and estrogens (Masson, '47). This constitutes an indirect confirmation of the work of Huzii ('38) and Fauvet ('42) who reported that lactation in castrated rats was inhibited by a combined treatment with progesterone and estrogens, although neither of the two hormones alone was active under the same experimental conditions. Unfortunately, the last two papers were not available to us. The purpose of the present work is to investigate the effect of various hormonal combinations on spayed lactating rats and to determine the role of the ovaries in lactation.

TABLE 1

Average growth rate and survival of sucklings following treatment of castrated lactating rats with steroids

GROUP	TREATMENT IN DAILY DOSE	NO. OF RATS	INITIAL BODY WT.	BODY WT. ON THE DAY			PERCENTAGE OF SURVIVALS ON THE DAY		
				5th	10th	15th	5th	10th	15th
I	Estradiol 10 γ	7	7.6	10.2	17	23.8	98	95	92
II	Estradiol 10 γ Progesterone 0.5 mg	8	7.2	11.1	17.3	24.2	100	96	94
III	Estradiol 10 γ Progesterone 5 mg	8	6.9	9.8	14.2	17.6	98	98	64
IV	Estradiol 10 γ Progesterone 10 mg	10	7.2	9.5	11.9	13.5	95	62	32
V	Estradiol 5 γ Progesterone 5 mg	4	7.3	10	13.4	14.3	100	95	95
VI	Estradiol 10 γ Testosterone 10 mg	5	7.2	10.4	16.6	18.5	97	77	66
VII	Estradiol 10 γ Dehydro-iso-androsterone 10 mg	4	7.9	11.1	19.7	22.9	100	100	100
VIII	.....	5	6.5	10	16	23.5	100	93	93

PROCEDURE

Female albino rates from the same colony, weighing 250–300 gm, and fed exclusively on Purina Fox Chow, were isolated as soon as pregnancy could be detected by palpation. On the day of delivery the number of sucklings per rat was reduced or brought to 6; 48 hours later the mothers were ovariectomized. Then the animals were divided into 8 groups and treated as indicated in table 1. The activity of the mammary gland was estimated from the growth curve and survival of the young. The steroids in oil solution were injected twice daily for a period of 15 days. On the 16th day, an autopsy was performed on the adult animals; the pituitary, adrenals, uterus and mammary glands were removed, weighed and examined misroscopically.

RESULTS

The results summarized in table 1 indicate the average body weight of the young rats at various intervals during lactation as well as the percentage of survivals. As previously shown, estradiol alone does not influence lactation in ovariectomized rats (compare groups I and VIII). However, in groups II, III and IV, which received increasing doses of progesterone together with the same amount of estradiol, a certain relationship exists between concurrent dosage with progesterone and inhibition of lactation. With a dose of 0.5 mg of progesterone no effect is noticeable but with 5 mg, and to a greater degree with 10 mg, a definite decrease in the growth of young and increased mortality is observed. In many respects the degree of inhibition is somewhat simlar to that obtained with estradiol, testosterone or androstenedione in non-spayed lactating rats (Barsantini and Masson, '47). Since in the most effective combination, the ratio estradiol/progesterone is equal to 1/1000, we decided in group V to change the absolute doses of the two hormones without modifying this ratio. The results were not as outstanding as in group IV but showed the same inhibition of growth without influencing the mortality. The groups VI and VII were added in order to study

the effect of two luteotropic and antilactogenic steroids, testosterone and dehydro-iso-androsterone given in combination with estradiol. The inhibition of lactation is absent with dehydro-iso-androsterone while with testosterone it corresponds approximately to that obtained with the combination of 10 γ of estradiol plus 5 mg of progesterone. This difference may be explained from the relative progestational activities of the two steroids: dehydro-iso-androsterone is without effect on the rabbit's uterus even at a dose of 50 mg while testosterone is active (Selye and Masson, '43).

The most interesting morphologic changes following these various treatments took place in the mammary gland of the animals treated with progesterone. In groups I, II and VIII the mammary gland was normal; acini and ducts were well developed and full of secretion (see fig. 1). In groups III and IV the mammary gland had a tendency to revert from the functional stage of lactation to the developmental stage seen during pregnancy. This involution was especially noticeable in group IV (see fig. 2) in which the acini were completely closed and devoid of secretion. These experiments proved that the antilactogenic steroids are not directly responsible for the inhibition of lactation. They first cause the formation of large coropora lutea which in turn, by their endocrine secretions (estrogen plus progesterone) transform the mammary gland from a stage of secretion into a stage of development either directly or through the pituitary.

These results are comparable to those obtained by Selye, Borduas and Masson ('42) and by Meites and Turner ('42). The first authors found a pronounced development of the mammary gland in spayed rats treated with various doses of estradiol (1–15 γ) and progesterone (1–5 mg). Milk secretion was also present except when the ratio of estradiol to progesterone was approximately equal to 1/2000. Similarly, Meites and Turner found that in guinea pigs the increase in pituitary lactogenic hormone usually observed following estrogenic treatment was prevented or reduced by simultaneous

administration of progesterone when the ratio of the two hormones was of the order of 1/2000.

Since it is known that removal or involution of large corpora lutea is followed by lactation, we ovariectomized pregnant rats in order to observe whether the ovary or the placenta is more important in the inhibition of lactation. In the rat abortion is a reasonably normal sequence to ovariectomy; however, since it occurs approximately 80 hours later (Zeiner, '43) there should be enough time for the initiation of lactation to take place.

Pregnant rats were divided into three groups. Animals of group I were ovariectomized at various periods of pregnancy; those of group II were ovariectomized also but were given sucklings approximately 10 days old; those of group III were kept as controls. Ovariectomy was performed from the 10th to the 15th day of pregnancy; the sucklings were changed twice a day in order to obtain an active and constant stimulation of the nipples. All the animals were killed 72 hours after the operation. Only a few animals of groups I and II aborted during this period, while in the others the fetuses were alive at autopsy. In all the animals of groups I and II, milk was secreted and could be expressed from the nipples. Histologic examination confirmed this observation. Acini were opened and dilated and ducts were full of secretions. No difference existed between the mammary glands of the rats of group I and those of group II. The status of the mammary gland in these two groups contrasted noticeably with that obtained in group III, which was characteristic of the pregnant stage (compare figs. 3–4 and 5–6). Therefore, even in the presence of the placenta, milk secretion can be initiated by removal of the ovaries. This may be explained by a change in the estrogen-progesterone ratio, and the fact that the gland can be stimulated to secrete at any period of its development.

If we accept the theory that large corpora lutea of pregnancy or those artificially produced by steroids or PMS are responsible for the inhibition of lactation, it is difficult to

understand lactation occurring in a pregnant rat. In order to study the effect of suckling during this condition, we gave rats at the same period of pregnancy as in the preceding experiment 6 young rats 10 days old which were changed twice daily. The mothers were sacrificed at various periods before delivery. Evidence of active suckling was provided by the pronounced development of the nipples. Presence of milk could never be detected by milking or by histologic examination of the mammary glands. It is difficult to comprehend the failure to initiate lactation in pregnant rats by this method, while lactation can occur normally in spite of a concomitant pregnancy. During the state of pregnancy-lactation, lactation takes place first, while placental development starts 7–15 days later depending on the number of young. During this initial period, the act of suckling influences the activity of the endocrine glands, particularly the pituitary and the ovaries. Desclin ('37) has shown that there is a definite decrease in the gonadotropic content of the pituitary and therefore a resulting decrease in the ovarian activity. Similar observations were made by Krehbiel ('41). It is possible that implantation impairs the normal endocrine secretions. This, however, does not interfere with the evolution of pregnancy and permits normal milk secretion. As a result of the delay in implantation, the large corpora lutea typical of pregnancy are formed at a time when the sucklings are ready to be weaned (Weichert and Schurgast, '42).

A recent paper by Meites and Turner ('47) on the induction of lactation during pregnancy by local injection of lactogenic hormone raises an important question of ovarian physiology: are the placental luteotropin and the pituitary prolactin identical, inasmuch as both are capable of maintaining the activity of corpora lutea? The observation of these authors suggests that they are different since lactation does not occur in spite of the presence of the placental luteotropin. It would be interesting to know why lactation was not elicited in pregnant animals when engaged in active suckling, since nipple stimulation by itself is sufficient to produce secretion

(Selye and McKeown, '34) or to maintain a high lactogen content of the pituitary (Meites and Turner, '42). Further experiments may prove that a difference in the threshold response of the ovaries and mammary gland is responsible.

ACKNOWLEDGMENT

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SUMMARY

Estradiol given in combination with progesterone to spayed lactating rats inhibits lactation when the ratio to progesterone is approximately of 1/1000. Testosterone but not dehydro-iso-androsterone given in combination with estradiol is also effective.

Ovariectomy in pregnant rats elicits milk secretion, while stimulation of the nipples is ineffective under the same conditions.

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PLATE 1

EXPLANATION OF FIGURES

1 Mammary gland of an untreated spayed rat.

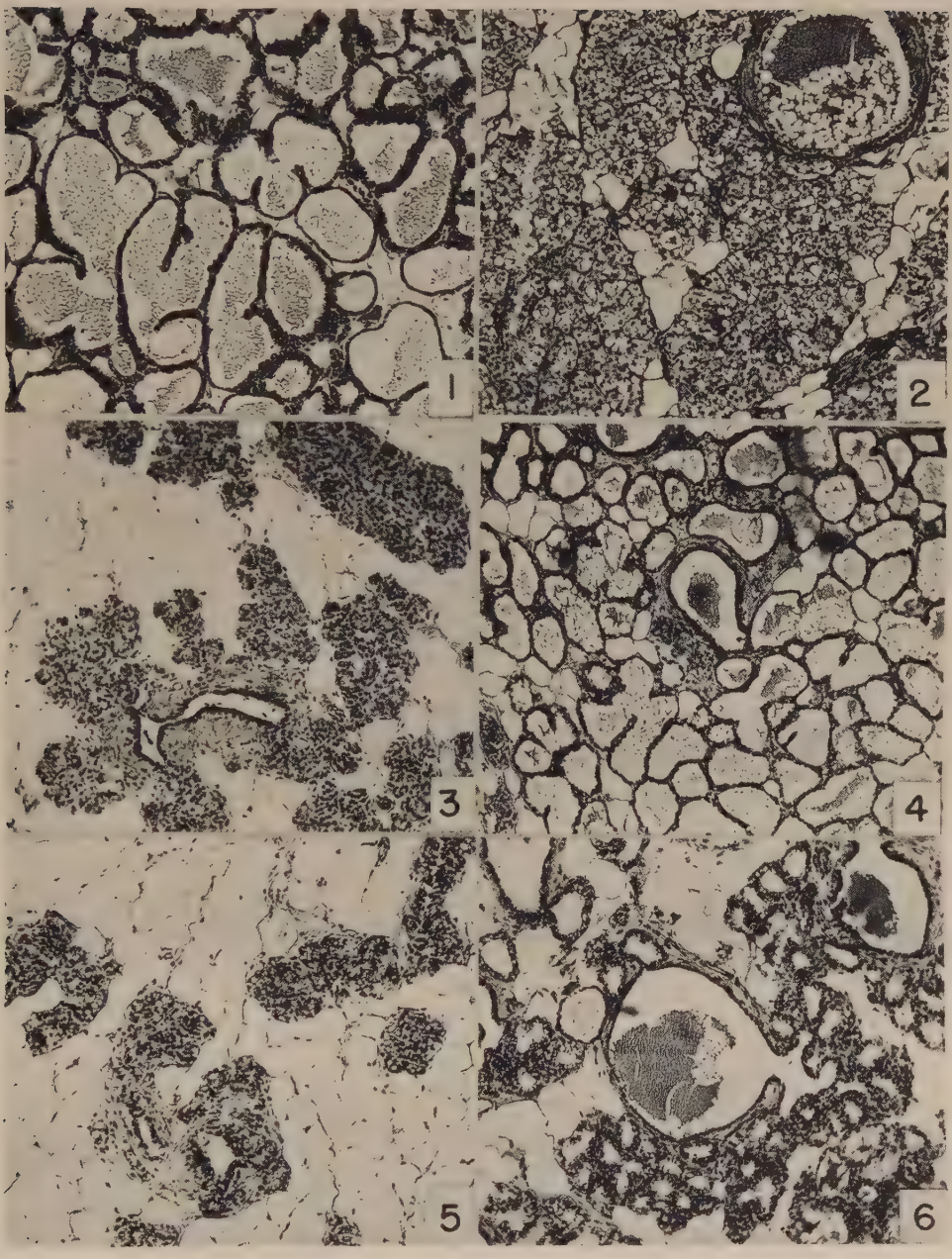
2 Mammary gland of a spayed rat treated with estradiol (10 γ/day) and progesterone (10 mg/day). Acini are closed and devoid of secretion.

3 Mammary gland of a normal rat on the 15th day of pregnancy.

4 Mammary gland of a rat spayed on the 12th day of pregnancy and autopsied 3 days later.

5 Mammary gland of a normal rat on the 9th day of pregnancy.

6 Mammary gland of a rat spayed on the 6th day of pregnancy and autopsied three days later.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THE RESULTS OF APPLICATION OF THE "ACID PHOSPHATASE" METHOD TO NERVOUS TISSUE AFTER FORMALIN FIXATION[1]

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FOUR FIGURES

The acid phosphatase method devised by Gomori ('39, '41) for the histological identification of sites of phosphatase activity constitutes an important procedure for the study of nervous tissue since it delineates all neural elements in a precise manner. However, the usefulness of the method has been markedly limited by the employment of acetone as a fixative as first recommended by Gomori ('41) and as practiced by various investigators. Although fixation and dehydration of fresh tissue by immersion in cold acetone has proved to be one of the best methods for the preservation of the catalyst, the slowness of penetration of acetone is a decided disadvantage, necessitating the use of pieces of tissue approximately 2 mm in thickness. Pieces of tissue thicker than this show irregular staining to such a degree that some investigators (Wolf, Kabat and Newman, '43) routinely discarded the first 0.1 mm of tissue sections. Furthermore, with this method of fixation, the necessity for the use of such thin pieces of tissue precludes the possibility of studies in which serial sections of relatively large blocks of tissue are necessary, a requirement which is often of primary consider-

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ation in neurological investigations. Perfusion with acetone or alcohol has not yielded uniformly good results, and this is not practical in the case of human material. Since nervous tissue prepared by the acid phosphatase method discloses not only nerve cells and their processes, but also other cellular elements, it has long been apparent that this procedure might be developed into a useful histological method for all nervous tissue, if it could be applied to the nervous system after fixation with formalin, which is the best general fixative for neural tissues.

The use of formalin as a fixative has been generally avoided in histochemical investigations employing the acid phosphatase method on the theory that the phosphatase is thereby inactivated. However, investigations carried out over a considerable period of time demonstrate clearly that this method yields a positive reaction in nervous tissue after formalin fixation, either by perfusion or immersion, provided the tissues are processed promptly after fixation and are incubated with the substrate for a longer period of time than is necessary in the case of acetone treated material. The results of the application of this method to certain parts of the nervous system of animals have been described briefly in previous reports by Smith and Luttrell ('47) and Smith ('48). More recent studies show that it is applicable to all parts of the nervous system of animals as well as to human autopsy material.

METHOD

The procedure in use in this laboratory with animal tissues is as follows. The animal is killed with ether and the blood is washed out by perfusion through the aorta with a small quantity of Ringer's solution. Fixation is then secured by perfusion with 20% formalin made up in Ringer's solution. Following this, blocks of tissue are removed and frozen sections are made, or the tissue is embedded by the paraffin method. Paraffin sections are mounted serially or as samples, the paraffin is removed with xylol and after hydration the

sections are incubated at 37.5°C. in the acid substrate made up according to the formula of Gomori ('41). The length of time of incubation is determined by processing sample sections at intervals. The intensity of the reaction can thus be readily controlled, and the optimum response attained for any particular tissue element. After processing, the sections are dehydrated in alcohol, cleared in tetrachlorethylene and mounted in synthetic resin dissolved in the clearing agent as

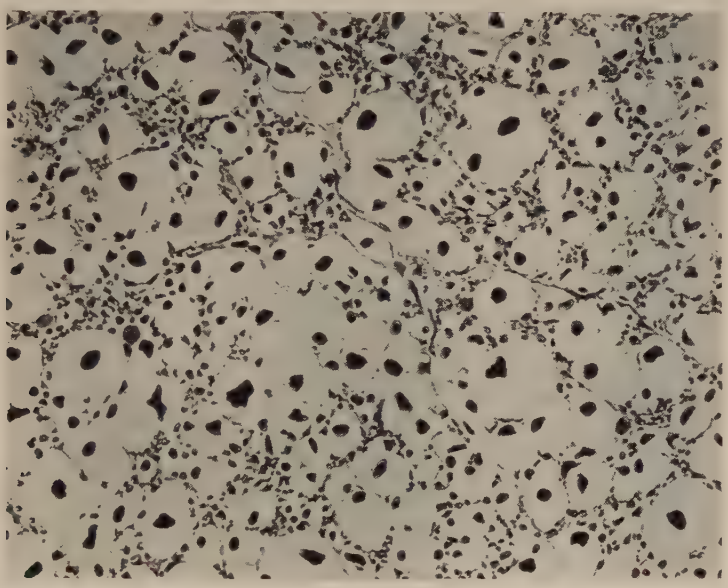

Fig. 1 Acid phosphatase reaction in the axons of spinal cord of cat. Myelin sheaths are colorless. × 500.

advocated by Gomori ('45) for sections prepared by his lipase method. Sections mounted in media containing xylol have often faded completely within a few weeks. Whether or not this was due to the xylol or to an impurity in it has not been determined.

RESULTS

In sections of spinal cord properly prepared, the nerve fibers of the white matter in cross section are darkly and

discretely stained; myelin sheaths are colorless (fig. 1). Even the finest fibers are discernible with a quality of distinctness comparable to that seen after silver impregnation. Nuclei of supporting cells show clear differentiation. Blood vessels stand out with clarity, the nuclei of the mural cells staining more darkly than their cytoplasm. Endothelial nuclei of the capillaries are clearly visible.

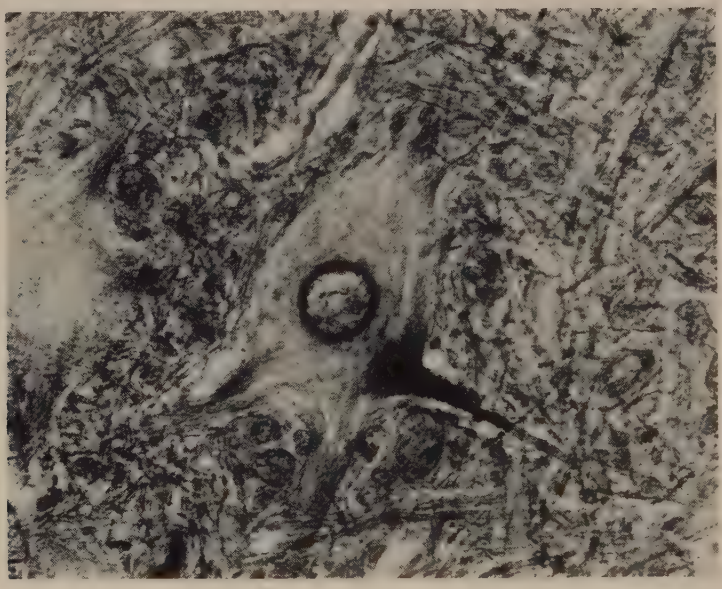

Fig. 2 Spinal somatic motor neuron. The perinuclear zone and the axon hillock show a more intense acid phosphatase reaction than other parts of the cell body. Cat. × 500.

The spinal gray matter usually exhibits a light brown background against which the cellular elements and the nerve fibers stand out in darker array. Nerve fibers coursing through the gray matter in all directions afford an interlacing background in the interstices of which lie nerve cells, presenting variations in appearance depending upon the type of cell and the intensity of the reaction. In general the nucleus stains darker than the surrounding cytoplasm and discloses irregularly stained masses (fig. 2). Often a darker ring sur-

rounds the nucleus, thus conspicuously delineating it from the adjacent less reactive cytoplasm. The nucleolus may stand out darkly stained or be unstained. At the base of the nerve cell processes the cytoplasm shows a more intense reaction and this extends out into both dendrites and axons, the broad base of the dendrites often presenting a fibrillar appearance. The axonal hillock is more darkly stained than any other part of the cell body. In some cells granules resembling Nissl

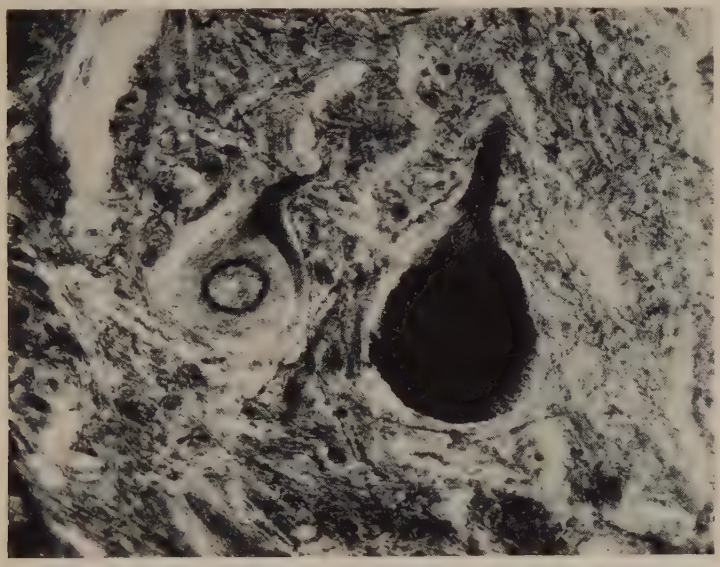

Fig. 3 Spinal somatic motor neurons. The cell on the right shows the increased phosphatase reaction 14 days after axonal section. Cat. × 500.

bodies are faintly visible against the more lightly stained cytoplasmic background. In addition to the nerve cells, the gray matter shows numerous glial cells, which appear as discrete granular nuclei without any visible surrounding cytoplasm.

The reaction in the substrate does not occur suddenly, but proceeds so gradually as to constitute progressive staining. The nerve fibers are the first to react. In both central and peripheral nervous systems the larger fibers react first

and for some hours are more heavily stained than the smaller ones. Nuclei of the interstitial glial tissue stain next, with the nuclei of the nerve cells staining later, followed by reaction in the neuronal cytoplasm. The length of time between staining of the fibers and the occurrence of reaction in the cell body is increased the longer the tissue has been left in formalin, so that after several days no reaction occurs in the cell body even after prolonged incubation, while the nerve fibers

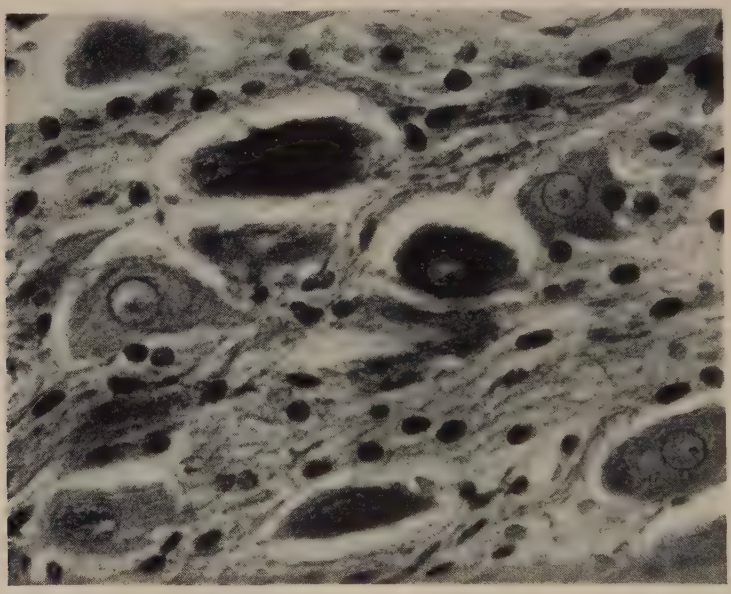

Fig. 4 Cells of the stellate ganglion of cat showing the acid phosphatase reaction. The darker cells show the increased reaction following axonal section. × 500.

still stain well. Immersion of the tissue in formalin for a longer time renders all of the tissue elements non-reactive. If the sections are processed for a longer time than is necessary for sharp delineation of the tissue elements, overstaining occurs. All tissue constituents then appear dark except the myelin sheaths which still remain colorless.

The acid phosphatase method is not only of value in the study of the architecture of normal neural tissue, but it

possesses usefulness in the study of tissues after various experimental procedures. This arises not only from the negative picture presented by degenerated pathways, but also from the discovery by Bodian and Mellors ('45) that the reaction is conspicuously increased in the cytoplasm of somatic motor neurons following axonal section. The striking contrast between the normal and the altered cell is shown in figure 3. Smith and Luttrell ('47) showed that a similar response occurs in both central and peripheral autonomic neurons (fig. 4). The acid phosphatase reaction thus provides a more precise method for neuronal localization than has previously existed.

In addition to its usefulness in the study of normal and experimental material in animals, the acid phosphatase method is applicable to the study of human post-mortem material fixed by immersion in formalin, provided the tissue is not left too long in the fixative. It thus becomes a valuable method in neuropathological, as well as in neurohistological and experimental neurological investigations.

COMMENT

The question naturally arises as to whether or not in the formalin fixed tissue the reaction is dependent upon an enzyme system or is the result of chemical reactions non-enzymatic in nature. This problem has recently been discussed by Lassek ('47) and by Bartelmez and Bensley ('47) with regard to peripheral nerves. Since the reaction is generally explained as depending upon the enzymatic disintegration of glycerophosphate with deposition of phosphate anions at the site of action and their combination with lead cations from the lead nitrate in the incubating subtrate, thus forming insoluble lead phosphate at the site of phosphatase activity, and subsequent replacement of the phosphate ions with sulfide, thus yielding lead sulfide, it might be possible to secure such a reaction in the absence of the enzyme if a dissociable lead compound were present in the tissues or if lead ions gained

entrance to the cells or their processes. However, placing the deparaffinized and hydrated sections in ammonium sulfide previous to incubation yields no reaction. The possibility that the incubation itself might lead to a release of loosely bound lead which then reacts to form lead sulfide seems disproved by the finding that no reaction occurs when the sections are incubated in the solution lacking the glycerophosphate. Heating fresh tissue or tissue that has previously been fixed in formalin or acetone to 100°C. for some time abolishes the reaction in a similar manner as does boiling frozen sections or paraffin sections that have been attached to the slide. The reaction is also inhibited by heating paraffin sections in an oven to 90° for 30 minutes. The addition of sodium fluoride so as to make 0.001 M concentration in the substrate inhibits the reaction. In addition, the reactiveness of the tissues gradually deteriorates so that tissues that have been in formalin for some weeks are not likely to show the reaction. Furthermore, other tissues that normally contain the enzyme such as spleen, kidney and liver not only react to the acid phosphatase process, but also show the enzyme quantitatively after formalin fixation, although in considerably reduced amounts. The evidence, therefore, is strongly in favor of the view that enzymatic activity is the basis of the reaction.

It is clearly evident that formalin fixation simultaneously reduces the reactivity of the tissues to the histochemical process and lessens the amount of enzyme as determined by the method of Huggins and Talalay ('45). From the histological point of view this is an advantage since the reaction is slowed enough to permit selective and progressive staining. The fact that frozen sections react much more quickly than paraffin ones indicates that activity is reduced by the various steps necessary for the preparation of paraffin sections. This has been made evident in the recent investigations of Stafford and Atkinson ('48) who found that enzymatic activity is progressively reduced at each stage in the preparation of the tissues. Since the sections show no reaction for some hours after being placed in the substrate, it may be that the first

step is one of release of reversibly bound formaldehyde from the formaldehyde enzyme complex, a reaction which has been shown to occur with some proteins (French and Edsall, '45). This view is supported by the finding that if sections prepared from formalin fixed tissues are placed in distilled water for some hours before incubation, a shorter time is required in the substrate. Then, however, the reaction appears more diffuse than when the sections are processed in the usual manner.

SUMMARY

The acid phosphatase method is applicable to formalin fixed tissues, clearly delineating the neural elements in both central and peripheral nervous systems. The essential requirements are the prompt processing of the tissues after fixation and incubation in the substrate for a longer time than is usual after acetone fixation. The evidence obtained in this investigation is entirely in favor of the view that the reaction in the formalin fixed tissues is enzymatic in character. Apart from the question as to whether or not the reaction is due to enzymatic activity, it constitutes a valuable method for the study of both normal and pathological tissues of the nervous system.

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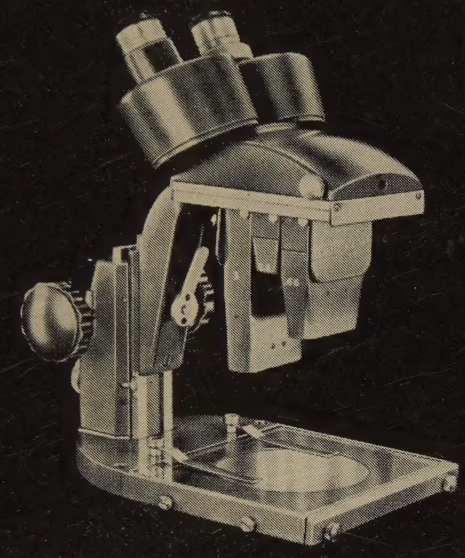

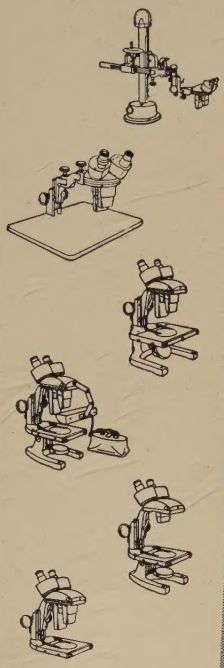

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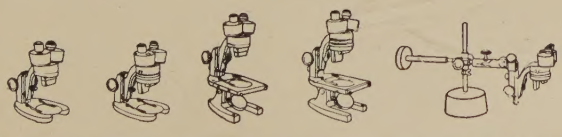

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